NEW AGENTS FOR TRANSFERRING NUCLEIC ACIDS, COMPOSITIONS CONTAINING THEM AND THEIR USES

The present invention relates to new transfer agents, the compositions containing them and their uses for the *in vitro*, *in vivo* or *ex vivo* transfer of nucleic acids into cells.

With the development of biotechnology, the possibility of effectively transferring nucleic acids into cells has become a basic technique with many biotechnological applications. It may involve the transfer of nucleic acids into cells *in vitro*, for example for producing recombinant proteins or, in the laboratory, for studying the regulation of gene expression, gene cloning or any other manipulation involving DNA. It may also involve the transfer of nucleic acids into cells *in vivo*, for example for producing vaccines, carrying out labeling studies or also carrying out therapeutic approaches. It may also involve the transfer of genes into cells removed from an organism, with a view to their subsequent readministration, for example for creating transgenic animals.

Currently, the most widespread means for transferring genes into cells is the use of viral vectors. However, since the latter are not completely without risks, several other methods based on the use of synthetic vectors have been proposed. These synthetic vectors have two main functions: to complex

and compact the nucleic acid to be transfected, and to promote its passage across the plasma membrane and, possibly, across the two nuclear membranes.

Several families of synthetic vectors have been developed, such as for example polymers or biochemical vectors (consisting of a cationic protein associated with a cellular receptor) but considerable progress has in particular been accomplished in nonviral transfection, with the development of lipofectants, and more particularly cationic lipids. It has thus been demonstrated that cationic lipids, because of their overall positive charge, interfere spontaneously with DNA which is negative overall, forming nucleolipid complexes capable of fusing with cell membranes, and thus allow the intracellular release of the DNA.

Various types of cationic lipid have thus been synthesized: lipids containing a quaternary ammonium group (for example DOTMA, DOTAP, DMRIE, DLRIE, etc.), lipopolyamines such as, for example, DOGS, DC-Chol or the lipopolyamines disclosed in Patent Application WO 97/18185, lipids which combine both a quaternary ammonium group and a polyamine such as DOSPA, or lipids containing various other cationic entities, in particular amidinium groups (for example ADPDE, ADODE or the lipids of Patent Application WO 97/31935). In fact, the structural diversity of cationic lipids is partly a reflection of the

observation of the structure-activity relationship.

However, the use of these synthetic vectors still poses many difficulties, and their effectiveness remains to be improved. In particular, it would be desirable to be able to have non-cationic or less cationic vectors, this being for various reasons:

- the complexes formed between the nucleic acid and the transfer agents, because of their overall positive charge, tend to be taken up by the reticuloendothelial system, which induces their elimination,
- because of the overall positive charge of the complexes formed, the plasma proteins tend to adsorb to their surface, and a loss of the transfection power results therefrom,
- in a context of local injection, the presence of a considerable overall positive charge prevents the diffusion of the nucleic acid complexes outside the site of administration, since the complexes are adsorbed onto the extracellular matrices. The complexes can, therefore, no longer reach the target cells, which, consequently, causes a decrease in the effectiveness of transfer with respect to the injected amount of complexes,
- and finally, many of those participating in the domain of nonviral transfection of genes have pointed out that cationic lipids or polymers have an inflammatory effect.

Moreover, the stable formulation of the

synthetic vectors developed up until now, at low charge ratios, is in general difficult, or even impossible, and it has also been noted that at a low charge ratio, the effectiveness of transfer is often poor (Pitard et al., PNAS USA, 94, pp. 14412-14417, 1997). In the remainder of the text, the term "charge ratio" means the ratio of the positive charges of the transfer agent to the negative charges of the DNA. This ratio is often expressed in nmol of transfer agent per µg of DNA.

These are the problems which the new transfecting agents developed by the applicant, and which form the subject of the present invention, propose to resolve. Specifically, their particular structure forms a hydrophobic anchor linked, firstly, to a polycation which allows the formation of complexes with the nucleic acids and, secondly, to at least one hydrophilic head which makes it possible to decrease the apparent overall charge density of these transfecting agents with respect to the cationic lipids or polymers conventionally used in nonviral transfection. The presence of at least one hydrophilic head creates a sort of "charge barrier" by decreasing the zeta potential of the complexes formed with the nucleic acid. Thus, said complexes appear less cationic to the organism, with the beneficial consequences which ensue therefrom. In addition, it has been shown that the transfecting agents according to the present invention are particularly advantageous from a

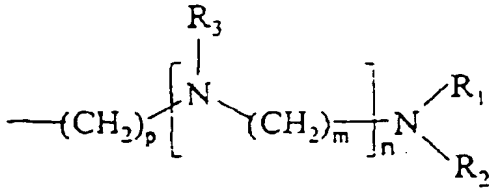
physicochemical point of view, since they are particularly stable when they are brought into contact with nucleic acids at low charge ratios.

Thus, a first subject of the invention relates to new agents for transfecting nucleic acids, which comprise a hydrophobic spacer chemically linked, firstly, to a polycation and, secondly, to at least one hydrophilic substituent.

The polycation makes it possible to form complexes with the nucleic acids by interactions with the anionic charges of the nucleic acids. The hydrophobic spacer has a double function. Firstly, it allows passage through the cell membranes and, secondly, it makes the complexes formed with the nucleic acids viable in biological medium. Specifically, the hydrophobic spacer creates a physical constraint on complexes which makes it possible to protect the nucleic acids against the outside medium. The hydrophobicity required for the complexes to be viable can be easily determined by those skilled in the art, using ordinary research methods or using the conventional method of trial and error. Finally, the presence of the hydrophilic substituent(s) makes it possible to decrease the zeta potential of the complexes formed, which makes said complexes appear less cationic to the outside medium.

For the purposes of the invention, the polycation is a linear or branched, polycationic

molecule capable of associating with nucleic acids. For the purpose of the invention, the expression "association with the nucleic acid" is intended to mean any type of attachment, such as for example covalent attachments, electrostatic or ionic interactions, hydrogen bridges, etc. Preferably the polycation is a linear or branched polyamine, each amino group being separated by one or more methylene groups. Optionally, the polyamine can also be substituted with other cationic functions, for example amidinium or guanidinium groups, cyclic guanidines, etc. It may in particular be a polycation as defined in Patent Applications WO 96/17823, WO 97/18185, WO 97/31935, WO 98/54130 or WO 99/51581, and more generally in the entire literature concerning cationic lipid structures, known to those skilled in the art. According to a preferred aspect of the invention, the polycation represents a polyamine of general formula (II):



(II)

in which:

- R_1, R_2 and R_3 represent, independently of each other, a hydrogen atom or a $(CH_2)_qNR'R''$ group with q an integer possibly ranging from 1 to 6, this being independently

among the various R_1, R_2 and R_3 groups, it being understood that at least one of R_1, R_2 and R_3 is other than a hydrogen atom,

- R' and R" represent, independently of each other, a hydrogen atom or a $(CH_2)_qNH_2$ group with q defined as above,

- m represents an integer between 1 and 6, and

- n and p represent, independently of each other, integers between 0 and 6, with, when n is greater than or equal to 2, m being able to have different values and R_3 different meanings within the general formula (II) and, when n is equal to 0, at least one of the R_1 and R_2 substituents is other than a hydrogen atom.

Other possible polycations can also be chosen from spermine, spermidine, cadaverine, putrescine, hexamethylenetetramine (hexamine), methacrylamidopropyltrimethylammonium chloride (AMBTAC), 3-acrylamido-3-methylbutyltrimethylammonium chloride (AMBTAC), polyvinylamines, polyethyleneimines, or ionenes (references: Barton et al., *Comprehensive Organic Chemistry*, Vol. 2, Ed. Pergamon Press, p. 90; *Encyclopedia of Polymer Science and Engineering*, 2nd Edition, Ed. Wiley Interscience, Vol. 11, p. 489; Mahler and Cordes, *Biological Chemistry*, Harper International Edition, p. 124).

The hydrophobic spacer can have very varied structures as long as it provides a hydrophobicity

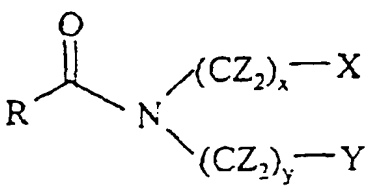
sufficient to allow the protection of the nucleic acids and the passage through membranes. This sufficient hydrophobicity can be determined by those skilled in the art, using ordinary research methods. According to a preferred variant of the invention, the hydrophobic spacer consists of 2 or 3 hydrocarbon-based linear fatty chains (i.e. between 10 and 20 carbon atoms per chain, and preferably, 12, 14, 15, 16, 17 or 18 carbon atoms per chain, each chain possibly being of different length). According to another variant, the hydrophobic spacer consists of a very long hydrocarbon-based linear fatty chain, i.e. comprising between 20 and 50 carbon atoms, and preferably between 40 and 50 carbon atoms, and even more preferably between 44 and 50 carbon atoms.

Hydrophilic substituents which are suitable are, for example, chosen from hydroxyl or amino substituents, polyols, sugars or hydrophilic peptides. The term "polyol" is intended to mean any linear, branched or cyclic hydrocarbon-based molecule comprising at least two hydroxyl functions. By way of example, mention may be made of glycerol, ethylene glycol, propylene glycol, tetritols, pentitols, cyclic pentitols (or quercitols), hexitols such as mannitol, sorbitol, dulcitols, cyclic hexitols or inositols, etc. (Stanek et al., The Monosaccharides Academic Press, pp. 621-655 and pp. 778-855).

According to an advantageous variant, the

transfer agents according to the invention comprise at least one hydrophilic substituent which is a sugar. For the purpose of the invention, the term "sugar" is intended to mean any molecule consisting of one or more saccharides. By way of example, mention may be made of sugars such as pyranoses and furanoses, for example glucose, mannose, rhamnose, galactose, fructose or maltose, lactose, saccharose, sucrose, fucose, cellobiose, allose, laminarabiose, gentiobiose, sophorose, melibiose, etc. Preferably, the sugar(s) is (are) chosen from glucose, mannose, rhamnose, galactose, fructose, lactose, saccharose and cellobiose. In addition, they may also be "complex" sugars, i.e. several sugars covalently coupled to each other, each sugar preferably being chosen from the list mentioned above. By way of suitable polysaccharides, mention may be made of dextran, α-amylose, amylopectin, fructans, mannans, xylans and arabinans. Certain preferred sugars can also interact with cellular receptors, such as for example certain types of lectin.

More particularly, the transfer agents according to the invention can be represented by the general formula (I):



(I)

for which:

- R represents a polycation,
- Z represents a hydrogen atom or a fluorine atom, the various Zs being independent of each other, and
- either x and y, independently of each other, represent integers between 10 and 22 inclusive, and X and Y, independently of each other, represent a hydrogen atom, an -OAlk group in which Alk represents a straight or branched alkyl containing 1 to 4 carbon atoms, a hydroxyl group, an amino group, a polyol, a sugar, a hydrophilic or non-hydrophilic peptide, or an oligonucleotide, it being understood that at least one of the X and Y substituents represents a hydrophilic group chosen from hydroxyl groups, amino groups, polyols, sugars or hydrophilic peptides,
- or x is equal to 0 or 1, y is an integer between 20 and 50, X is either a hydrogen atom or an -OAlk group in which Alk represents a straight or branched alkyl containing 1 to 4 carbon atoms, and Y is a hydrophilic group chosen from hydroxyl groups, amino groups, polyols, sugars or hydrophilic peptides.

For the purpose of the invention, the polycation, the polyols and the sugars of general formula (I) are as defined above.

The terms x and y are defined in general formula (I) so as to have any value between 10 and 22 inclusive or between 20 and 50 inclusive, depending on the cases. Preferably, x and y, independently of each other, are between 12 and 18 inclusive. More

preferably, x and y have the value, independently of each other, 14, 15, 16, 17 or 18. When x is equal to 0 or 1, then y is preferably between 30 and 50, or between 40 and 50. More preferably y is between 44 and 50.

For the purpose of the invention, the term "oligonucleotide" is intended to mean chains containing one or more nucleotides, deoxynucleotides, ribonucleotides and/or deoxyribonucleotides which are monomeric units differing from each other by the presence of bases which can be chosen from adenine, guanine, cytosine, thymidine or uracil [see Lehninger Biochimie, Flammarion Medecine Sciences, 2nd edition, p. 305-329]. Because of their base-pair-forming property, oligonucleotides are widely used in molecular biology, for example as linkers (attachment molecule) or as probes.

Moreover, oligonucleotides can be used in the form of conjugates, i.e. coupled to one or more other molecules having distinct properties. By way of example, mention may be made of the coupling of an oligonucleotide with a reactive chemical group, with fluorescent or chemiluminescent groups, or with groups capable of enhancing intermolecular interactions so as to promote entry into cells. Such conjugates, which are described in Bioconjugate Chemistry [John Goodchild, *Conjugates of Oligonucleotides and Modified Oligonucleotides: a Review of their Synthesis and*

properties, Vol. 1, No. 3, 1990, pp. 165-187], have many uses and advantages, such as for example the capacity to improve the entry of complexes into cells, to decrease the degree of degradation by nucleases, to increase the stability of the complex in question, to monitor the fate of oligonucleotides in an organism, etc. Thus, the oligonucleotide(s), when it (they) is (are) grafted onto the transfer agents according to the present invention, make it possible to provide said transfer agents with an additional property (for example targeting, labeling, etc. properties).

The oligonucleotides can be obtained according to conventional methods known to those skilled in the art, and it is also possible to synthesize modified oligonucleotides according to the methods described in Bioconjugate Chemistry, John Goodchild, *Conjugates of Oligonucleotides and Modified Oligonucleotides: a Review of their Synthesis and properties*, Vol. 1, No. 3, 1990, pp. 165-187 or in Tetrahedron, Beaucage et al., *The Synthesis of Modified Oligonucleotides by the Phosphoramiditeb Approach and Their Application*, Vol. 49, No. 28, pp. 6123-6194, 1993.

For the purpose of the invention, the term "peptide" is intended to mean chains containing one or more amino acids linked to each other via attachments of a peptide nature [Lehninger Biochimie, Flammarion Medecine Sciences, 2nd edition]. They may be the 20

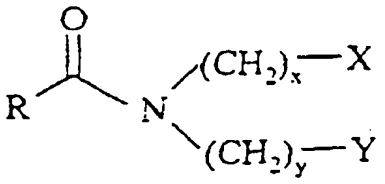
"conventional" amino acids, i.e. those commonly found in the composition of proteins (alamine, valine, leucine, isoleucine, proline, phenylalamine, tryptophan, methionine, aspartic acid, glutamine, lysine, arginine, histidine, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamic acid), or they may also be the "rare" amino acids, such as for example 4-hydroxyproline, desmosine, 5-hydroxylysine, N-methyllysine, 3-methylhistidine, isodesmosine, etc. Finally, they may also be the amino acids which appear in various cells or diverse tissues in free or combined form, and which derive, in general, from α-amino acids (for example β-alamine, γ-aminobutyric acid, homocysteine, ornithine, canavanine, djenkalic acid, β-cyanoalamine, etc.). Such peptides can, for example, allow the targeting of certain cell types. In this context, mention may, for example, be made of RGD or NLS peptides. They may also be peptide sequences having labeling properties, i.e. allowing identification, for example using analytical techniques such as fluorescence spectrometry, infrared spectrometry, nuclear magnetic resonance (NMR), etc. In this respect, mention may, for example, be made of linear or cyclic, peptide or pseudopeptide sequences containing the Arg-Gly-Asp (Arginine-Glycine-Aspartic Acid) recognition epitope of the primary and/or secondary receptors for adhesion proteins of the integrin type.

The peptides according to the invention can also be substituted on one or more of their functional groups, for example on the α carboxyl, on the α amine function and/or on the functional groups of the side chain of each of the amino acids. By way of example, mention may be made of substitutions with linear, branched or cyclic, saturated or unsaturated, aliphatic groups containing 1 to 24 carbon atoms, such as, for example, cholesteryl, arachidonyl or retinoyl radicals, or mono- or polyaromatic groups such as, for example, substituted or unsubstituted benzyloxycarbonyl, benzyl ester, or rhodaminyl derivatives. The advantage of such substitutions lies in the modification of the chemical and possibly biological properties of said peptides, for example in order to label them.

When said peptides are used as a hydrophilic substituent, they are chosen from hydrophilic peptides, i.e. peptides consisting only of hydrophilic amino acids, or those which are partially composed of hydrophilic amino acids and the composition of which makes them hydrophilic overall.

According to a preferred variant of the invention, the Z groups all represent hydrogen atoms.

According to a more particularly advantageous aspect of the invention, the transfer agents are of general formula (III):



(III)

for which:

- R represents a polycation, and
- either x and y, independently of each other, represent integers between 10 and 22 inclusive, and X and Y, independently of each other, represent a hydrogen atom or a sugar, it being understood that at least one of the X and Y substituents represents a sugar,
- or x is equal to 0 or 1, y is an integer between 20 and 50, X is a hydrogen atom and Y is a sugar.

For the purpose of the invention, the polycation, the sugars and x and y in general formula (III) are as defined above for general formula (I).

Transfer agents which are more particularly preferred are of general formula (III), and x and y, independently of each other, represent integers between 10 and 22 inclusive, and one of X and Y represents a hydrogen atom and the other a sugar. According to another advantageous variant, the transfer agents according to the invention are of general formula (III), and x is equal to 0, y is an integer between 40 and 50, X represents a hydrogen atom and Y

is a sugar.

It is understood that the present invention also relates to the isomers of the products of general formula (I), when they exist, and also to mixtures thereof or salts thereof.

In particular, the compounds of the invention can be in the form of nontoxic and pharmaceutically acceptable salts. These nontoxic salts comprise salts with inorganic acids (for example hydrochloric, sulfuric, hydrobromic, phosphoric and nitric acid), with organic acids (acetic, propionic, succinic, maleic, hydroxymaleic, benzoic, fumaric, methanesulfonic or oxalic acid) with inorganic bases (sodium hydroxide, potassium hydroxide, lithium hydroxide, lime) or with organic bases (tertiary amines such as triethylamine, piperidine, benzylamine).

According to the invention, the preparation of the products of general formula (I) is carried out using the following steps:

1) Firstly, an alkyl chain with x carbon atoms (x being defined as above), containing a hydroxyl function and an ester function, is prepared by opening a corresponding lactone. The reaction is generally carried out in an alcohol, at basic pH and at a temperature of between -10°C and room temperature. By way of example, the alcohol can be methanol or ethanol.

2) Then, the X group is attached to the difunctional alkyl chain obtained in the preceding

step. When X represents a sugar, a condensation is carried out in a chlorinated solvent, such as for example dichloromethane or chloroform, and in the presence of a Lewis acid, at a temperature of between -5°C and 10°C. The Lewis acid can, for example, be chosen from tin chloride, iron chloride, p-toluenesulfonic acid (tsOH), trimethylsillyltrifluoromethanesulfonic (TMStf), boron trifluoride etherate, etc. [Kazunobu Toshima et al., *Recent Progress in O-glcosilation Methods and its Application to Natural Products Synthesis*, Chem. Rev. 1993, Vol. 93, pp. 1503-1531].

When X represents a hydrophilic or non-hydrophilic peptide group, peptide coupling is carried out according to conventional methods (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springe-Verlag) or using any similar method known to those skilled in the art. In particular, the reaction is generally carried out in the presence of a non-nucleophilic base in suitable aprotic solvents, at a temperature of between 0 and 100°C, the pH being adjusted to between 9 and 11. By way of example, chloroform, dimethylformamide, methylpyrrolidone, acetonitrile, dichloromethane, toluene or benzene can be used as a solvent. The non-nucleophilic bases used are preferably tertiary amines, calcium carbonate or sodium dicarbonate. Even more preferably, the bases used are tertiary amines

such as, for example, triethylamine (TEA) or N-ethyldiisopropylamine. Advantageously, the peptide coupling is carried out between 0 and 50°C, and preferably between 10 and 30°C.

When it is desired that X represents a hydroxyl group, this step is not carried out.

When X represents an amino group, the reaction is carried out by nucleophilic substitution according to conventional methods known to those skilled in the art, which enable an amine to be obtained from an alcohol.

When X represents an -OAlk group, alkylation of the alcohol function is carried out according to conventional methods known to those skilled in the art or according to similar methods. For example, a diazo compound of general formula Alk-N_2 can be reacted optionally in the presence of a catalyst such as HBF_4 or of silica gel. It is also possible to operate under Williamson Reaction conditions, which consists in reacting, in basic medium, a compound of general formula Alk-Hal in which Hal represents a halogen atom such as chlorine, bromine or iodine, on the chain bearing an alcohol function.

The same Williamson-type reaction can also be used when it is desired that X represents a polyol.

Finally, when X represents an oligonucleotide, the latter is coupled to the difunctional chain according to known conventional

methods for covalently grafting an oligonucleotide. For example, said oligonucleotide can be grafted via a suitable linker (attachment molecule).

3) Thirdly, the ester function present on the difunctional chain is hydrolyzed to an acid function according to known methods. For example, the procedure can be carried out in basic medium in an alcohol with a high boiling point, at a temperature of between 50°C and the reflux temperature of the reaction mixture.

4) Then, a substituted or unsubstituted alkylamine chain of general formula (IV):

$$H_2N-(CH_2)_y-Y \qquad (IV)$$

in which y and Y are defined as above is coupled to the compound obtained in the preceding step, according to conventional peptide coupling methods (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springe-Verlag) or using any similar method known to those skilled in the art.

In particular, the reaction is generally carried out in the presence of a non-nucleophilic base in suitable aprotic solvents, at a temperature of between 0 and 100°C, the pH being adjusted to between 9 and 11. By way of example, chloroform, dimethyl-formamide, methylpyrrolidone, acetonitrile, dichloromethane, toluene or benzene can be used as a solvent. The non-nucleophilic bases used are preferably tertiary amines, calcium carbonate or sodium dicarbonate. Even more preferably, the bases used are

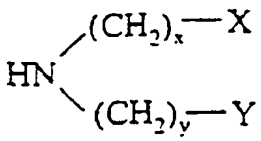
tertiary amines such as, for example, triethylamine (TEA) or N-ethyldiisopropylamine. Advantageously, the peptide coupling is carried out at between 0 and 50°C, preferably between 10 and 30°C.

The group of general formula (IV) is either commercially available, or it can be obtained by condensation of Y on the corresponding unsubstituted alkylamine, according to a method similar to that described above in 2).

5) The amide obtained in the preceding step is then reduced to an amine. The procedure for this is carried out according to conventional methods known to those skilled in the art. For example, the procedure is carried out in an anhydrous organic solvent such as anhydrous tetrahydrofuran, by reacting lithium aluminum hydride $LiAlH_4$. Other reducing agents which may be used are, for example, borane, borane in dimethyl sulfide (BH_3SMe_2), sodium borohydride/titanium tetrachloride ($NaBH_4$, $TiCl_4$), phosphorus oxychloride on zinc ($POCl_3/Zn$), phosphorus pentasulfide (P_4S_{10}) on Raney nickel, etc. [Richard C. Larock, Comprehensive Organic Transformations, VCH Publishers Inc., 1989]. The procedure can also be carried out by catalytic hydrogenation. Advantageously, the reduction is carried out by reacting lithium aluminum hydride $LiAlH_4$, in anhydrous tetrahydrofuran, at the reflux temperature of the mixture.

A compound of general formula (V) is thus

obtained:



(V)

for which X, Y, x and y are defined as above.

6) Finally, in a last stage, the acid derivative corresponding to the polycation R as defined above is coupled to the compound of general formula (IV) obtained in the preceding step, according to conventional peptide coupling methods (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springe-Verlag) or using any similar method known to those skilled in the art.

In particular, the reaction is generally carried out in the presence of a non-nucleophilic base in suitable aprotic solvents, at a temperature of between 0 and 100°C, the pH being adjusted to between 9 and 11. By way of example, chloroform, dimethylformamide, methylpyrrolidone, acetonitrile, dichloromethane, toluene or benzene can be used as a solvent. The non-nucleophilic bases used are preferably tertiary amines, calcium carbonate or sodium dicarbonate. Even more preferably, the bases used are tertiary amines such as, for example, triethylamine (TEA) or N-ethyldiisopropylamine. Advantageously, the peptide coupling is carried out at between 0 and 50°C, preferably between 10 and 30°C.

The acid derivatives corresponding to the polycation are commercially available.

According to another variant, the transfecting agents according to the present invention can be prepared by carrying out the following procedure:

1) Firstly, an alkyl chain with x carbon atoms (x being defined as above), containing a hydroxyl function and an ester function, is prepared by opening a corresponding lactone. The reaction is generally carried out in an alcohol, at basic pH and at a temperature of between -10°C and room temperature. By way of example, the alcohol can be methanol or ethanol.

2) Then, a substituted or unsubstituted alkylamine chain of general formula (IV):

$$H_2N-(CH_2)_y-Y \qquad (IV)$$

in which y and Y are as defined above in general formula (I) is coupled to this difunctional alkyl chain. The reaction is carried out at a temperature higher than the melting point of each product, possibly under vacuum. The reaction can also be carried out at the reflux temperature, in the presence of an alcoholic solvent. By way of example, the solvent can be methanol or ethanol. The procedure is carried out, for example, at a temperature of between 45°C and 60°C.

The reaction can also be carried out at the reflux temperature of the mixture, in the presence of

an alcohol, such as methanol, as a solvent. Another alternative consists in coupling the compound of general formula (IV) directly with the lactone (in this case, the first step of opening the lactone is not carried out).

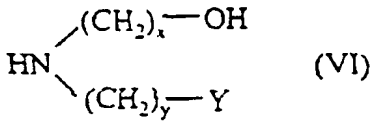
The group of general formula (IV) is either commercially available, or it can be obtained by condensation of Y on the corresponding unsubstituted alkylamine, according to a method similar to that described above.

3) The difunctional bicatenary amide obtained in the preceding step is then reduced to an amine. The procedure for this is carried out according to conventional methods. For example, the procedure is carried out in an anhydrous organic solvent such as anhydrous tetrahydrofuran, by reacting lithium aluminum hydride ($LiAlH_4$). Other reducing agents which may be used are, for example, borane, boron hydride dimethyl sulfide (BH_3-SMe_2), sodium borohydride/titanium tetrachloride ($NaBH_4$, $TiCl_4$), phosphorus oxychloride on zinc ($POCl_3$/Zn), phosphorus pentasulfide (P_4S_{10}) on Raney nickel, etc. [Richard C. Larock, Comprehensive Organic Transformations, VCH Publishers Inc., 1989]. The procedure can also be carried out by catalytic hydrogenation.

Advantageously, the reduction is carried out by reacting lithium aluminum hydride $LiAlH_4$, in anhydrous tetrahydrofuran, at the reflux temperature of

the mixture.

A compound of general formula (VI) is thus obtained:



(VI)

for which Y, x and y are defined as above.

4) Then, the X group is condensed on the amine of general formula (VI) obtained in the preceding step. The condensation is carried out according to methods similar to those previously described for the first synthetic pathway.

5) Finally, in a last stage, the acid derivative corresponding to the polycation R as defined above is coupled to the compound of general formula (VI) obtained in the preceding step, according to conventional peptide coupling methods (Bodanski M., *Principles and Practices of Peptides Synthesis*, Ed. Springe-Verlag) or using any similar method known to those skilled in the art.

In particular, the reaction is generally carried out in the presence of a non-nucleophilic base in suitable aprotic solvents, at a temperature of between 0 and 100°C, the pH being adjusted to between 9 and 11. By way of example, chloroform, dimethyl-formamide, methylpyrrolidone, acetonitrile, dichloromethane, toluene or benzene can be used as a solvent. The non-nucleophilic bases used are preferably

tertiary amines, calcium carbonate or sodium dicarbonate. Even more preferably, the bases used are tertiary amines such as, for example, triethylamine (TEA) or N-ethyldiisopropylamine. Advantageously, the peptide coupling is carried out at between 0 and 50°C, preferably between 10 and 30°C.

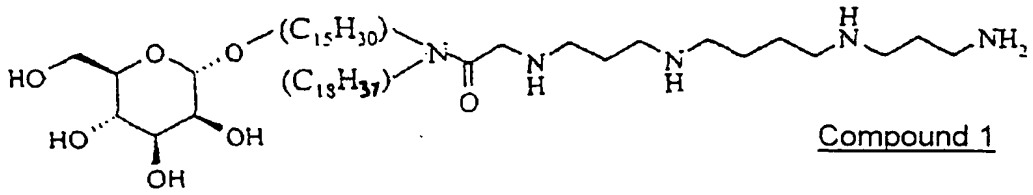
The acid derivatives corresponding to the polycation are commercially available.

Naturally, when the substituents of X, Y and/or of the polycation may interfere with the reaction, it is preferable to protect them beforehand with radicals which are compatible and which can be introduced and removed without affecting the rest of the molecule. The procedure for this is carried out according to conventional methods known to those skilled in the art, and in particular according to the methods described in T.W. GREENE, *Protective Groups in Organic Synthesis*, 2nd Edition, Wiley-Interscience, in McOMIE, *Protective Groups in Organic Chemistry*, Plenum Press (1973), or in Philip J Kocienski, *Protecting Groups*, Thieme.

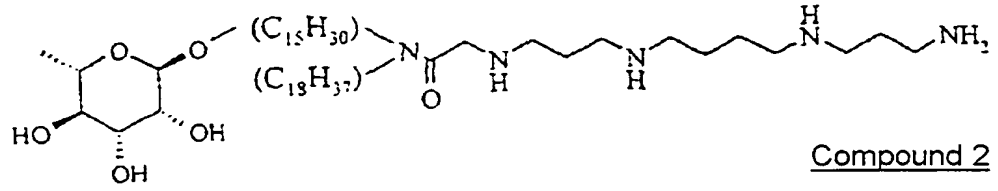
Moreover, each step of the preparation process can be followed, where appropriate, by steps for separating and purifying the compound obtained according to the methods known to those skilled in the art.

By way of an illustrative example of advantageous agents for transferring nucleic acids,

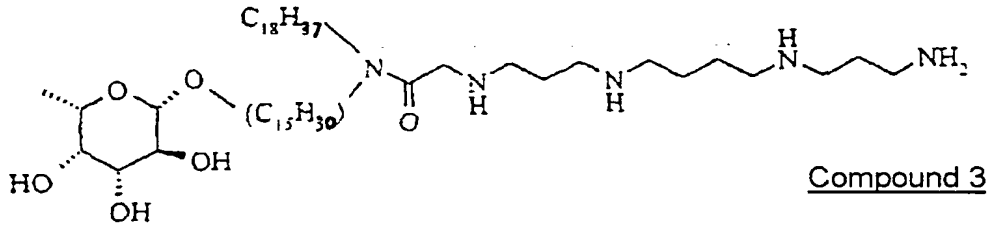
according to the invention, mention may be made of the following compounds:



Compound 1



Compound 2



Compound 3

Another subject of the invention relates to the compositions comprising an agent for transferring nucleic acids as defined above, and a nucleic acid. The respective amounts of each component can be easily adjusted by those skilled in the art as a function of the transfer agent used, of the nucleic acid and of the desired applications (in particular of the type of cells to be transfected).

For the purpose of the invention, the term "nucleic acid" is intended to mean both a deoxyribonucleic acid and a ribonucleic acid. They may

be natural or artificial sequences, and in particular genomic DNA (gDNA), complementary DNA (cDNA), messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), hybrid sequences or synthetic or semi-synthetic sequences, or oligonucleotides which may or may not be modified. These nucleic acids can be of human, animal, plant, bacterial, viral, etc. origin. They can be obtained using any technique known to those skilled in the art, and in particular by screening libraries, by chemical synthesis, or using mixed methods including the chemical or enzymatic modification of sequences obtained by screening libraries. They may be chemically modified.

With regards more particularly to deoxyribonucleic acids, they can be single- or double-stranded, as well as short oligonucleotides or longer sequences. In particular, the nucleic acids conventionally consist of plasmids, vectors, episomes, expression cassettes, etc. These deoxyribonucleic acids can carry an origin of replication which may or may not be functional in the target cell, one or more marker genes, sequences which regulate transcription or replication, genes of therapeutic interest, antisense sequences which may or may not be modified, regions for binding to other cellular components, etc.

Preferably, the nucleic acid comprises one or more genes of therapeutic interest under the control of regulatory sequences, for example one or more promoters

and a transcriptional terminator which are active in the target cells.

For the purpose of the invention, the term "gene of therapeutic interest" is intended to mean, in particular, any gene encoding a protein product having a therapeutic effect. The protein product thus encoded can in particular be a protein or a peptide. This protein product can be exogenous, homologous or endogenous with respect to the target cell, i.e. a product which is normally expressed in the target cell when the latter has no pathological condition. In this case, the expression of a protein makes it possible, for example, to overcome insufficient expression in the cell or the expression of a protein which is inactive or weakly active because of a modification, or to overexpress said protein. The gene of therapeutic interest can also encode a mutant of a cellular protein, having increased stability, modified activity, etc. The protein product can also be heterologous with respect to the target cell. In this case, an expressed protein can, for example, supplement or provide an activity which is deficient in the cell, allowing it to combat a pathological condition, or to stimulate an immune response.

Among the therapeutic products for the purpose of the present invention, mention may be made more particularly of enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF,

etc. (FR 92/03120), growth factors, neurotransmitters or precursors thereof or synthetic enzymes thereof, trophic factors (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleiotrophin, etc.), apolipoproteins (ApoAI, ApoAIV, ApoE, etc., FR 93/05125), dystrophin or a minidystrophin (FR 91/11947), the CFTR protein associated with cystic fibrosis, tumor suppressor genes (p53, Rb, Rap1A, DCC, k-rev, etc., FR 93/04745), genes encoding factors involved in clotting (factors VII, VIII and IX), genes involved in DNA repair, suicide genes (thymidine kinase, cytosine deaminase), the genes for hemoglobin or for other carrier proteins, metabolic enzymes, catabolic enzymes, etc.

The nucleic acid of therapeutic interest can also be an antisense sequence or gene, the expression of which in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can, for example, be transcribed, in the target cell, into RNAs which are complementary to cellular mRNAs and thus block their translation into protein, according to the technique described in patent EP 140 308. The therapeutic genes also comprise the sequences encoding ribozymes, which are capable of selectively destroying target RNAs (EP 321 201).

As indicated above, the nucleic acid can also contain one or more genes encoding an antigenic peptide capable of generating an immune response in humans or

animals. In this particular embodiment, the invention allows the production of vaccines or the carrying out of immunotherapeutic treatments applied to humans or to animals, in particular against microorganisms, viruses or cancers. They may in particular be antigenic peptides specific for the Epstein-Barr virus, for the HIV virus, for the hepatitis B virus (EP 185 573), for the pseudorabies virus, for the syncitia forming virus or for other viruses, or antigenic peptides specific for tumors (EP 259 212).

Preferably, the nucleic acid also comprises sequences allowing the expression of the gene of therapeutic interest and/or of the gene encoding the antigenic peptide, in the desired cell or organ. They may be sequences which are naturally responsible for the expression of the gene in question when these sequences are capable of functioning in the infected cell. They may be sequences of different origin (responsible for the expression of other proteins, or even synthetic). In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell the infection of which is desired. Similarly, they may be promoter sequences derived from the genome of a virus. In this regard, mention may be made, for example, of the E1A, MLP, CMV, RSV, etc. gene promoters. In addition, these expression sequences can be modified by adding activation sequences, regulatory

sequences, etc. The promoter may also be inducible or repressible.

Moreover, the nucleic acid can also contain, in particular upstream of the gene of therapeutic interest, a signal sequence which directs the therapeutic product synthesized into the secretory pathways of the target cell. This signal sequence may be the natural signal sequence of the therapeutic product, but it may also be any other functional signal sequence or an artificial signal sequence. The nucleic acid may also contain a signal sequence which directs the therapeutic product synthesized toward a particular compartment of the cell.

The compositions according to the invention can also contain one or more adjuvants capable of combining with the transfer agent/nucleic acid complexes and of improving the transfecting power thereof. In another embodiment, the present invention therefore relates to compositions comprising a nucleic acid, an agent for transferring nucleic acids as defined above and at least one adjuvant capable of combining with the transfer agent/nucleic acid complexes and of improving the transfecting power thereof. The presence of this type of adjuvant (lipids, peptides or proteins, for example) may advantageously make it possible to increase the transfecting power of the compounds. In this respect, the compositions of the invention can comprise, as an adjuvant, one or more

neutral lipids.

More preferably, the neutral lipids used in the context of the present invention are lipids with two fatty chains. Particularly advantageously, natural or synthetic lipids, which are zwitterionic or lacking ionic charge under physiological conditions, are used. They can be chosen more particularly from dioleoylphosphatidylethanolamine (DOPE), oleoylpalmitoylphosphatidylethanolamine (POPE), di-stearoyl, -palmitoyl, -myristoylphosphatidyl-ethanolamines and also the derivatives thereof which are N-methylated 1 to 3 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebrosides (such as in particular galactocerebrosides), sphingolipids (such as in particular sphingomyelins) or asialogangliosides (such as in particular asialoGM1 and GM2).

These various lipids can be obtained either by synthesis or by extraction from organs (for example: brain) or from eggs, using conventional techniques known to those skilled in the art. In particular, the extraction of natural lipids can be carried out by means of organic solvents (see also Lehninger, Biochemistry).

More recently, the Applicant has demonstrated that it is also particularly advantageous to use, as an adjuvant, a compound which is involved possibly directly in the condensation of said nucleic acid, such

as those described in patent application WO 96/25508. The presence of such a compound in a composition according to the invention makes it possible to reduce the amount of transfecting agent, with the beneficial consequences which ensue therefrom from the toxicological point of view, without any damaging effect on the transfecting activity. The expression "compound which is involved in the condensation of nucleic acid" is intended to define a compound which compacts, directly or indirectly, the nucleic acid. More precisely, this compound can either act directly on the nucleic acid to be transfected, or can be involved at the level of an additional compound which, itself, is direcly involved in the condensation of this nucleic acid. Preferably, it acts directly on the nucleic acid. In particular, the precompacting agent can be any polycation, for example polylysine. According to a preferred embodiment, the agent which is involved in the condensation of the nucleic acid is derived, as a whole or in part, from a protamine, from a histone or from a nucleolin, and/or from a derivative thereof. Such an agent can also consist, as a whole or in part, of peptide units (KTPKKAKKP)$_n$ (SEQ ID NO:1) and/or (ATPAKKAA)$_n$ (SEQ ID NO:2), the number of units possibly ranging between 2 and 10. In the structure of the compound according to the invention, these units may be repeated continuously or discontinuously. They may thus be separated by linkages of a biochemical nature, for

example by one or more amino acids, or of a chemical nature.

Preferably, the compositions of the invention comprise from 0.01 to 20 equivalents of adjuvant per equivalent of nucleic acid in mol/mol, and more preferably from 0.5 to 5.

In a particularly advantageous embodiment, the compositions according to the present invention also comprise a targeting element which makes it possible to orient the transfer of the nucleic acid. This targeting element may be an extracellular targeting element which makes it possible to orient the transfer of the DNA toward certain desired cell types or tissues (tumor cells, hepatic cells, hematopoietic cells, etc.). It may also be an intracellular targeting element which makes it possible to orient the transfer of the nucleic acid toward certain preferred cellular compartments (mitochondria, nucleus, etc.). The target element can be linked to the agent for transferring nucleic acids according to the invention, or also to the nucleic acid as specified above. When the targeting element is linked to the agent for transferring nucleic acids, of general formula (I), this element preferably constitutes one of the X or Y substituents.

Among the targeting elements which can be used in the context of the invention, mention may be made of sugars, peptides, proteins, oligonucleotides, lipids, neuromediators, hormones, vitamins or

derivatives thereof. Preferably, they are sugars, peptides or proteins such as antibodies or antibody fragments, ligands of cell receptors or fragments thereof, receptors or receptor fragments, etc. In particular, they are ligands of growth factor receptors, of cytokine receptors or of cellular lectin-type receptors, or RGD sequence-containing ligands with an affinity for the receptors for adhesion proteins such as integrins. Mention may also be made of the receptors for transferrin, for HDLs and LDLs, or the folate transporter. The targeting element can also be a sugar which makes it possible to target lectins such as the receptors for asialoglycoproteins or for sialidized species, such as sialyl Lewis X, or an Fab antibody fragment or a single chain antibody (ScFv).

The association of the targeting elements with the nucleolipid complexes can be formed using any technique known to those skilled in the art, for example by coupling to a hydrophobic portion or to a portion which interacts with the nucleic acid of the transfer agent according to the invention, or to a group which interacts with the transfer agent according to the invention or with the nucleic acid. The interactions in question can be, according to a preferred mode, ionic or covalent in nature.

A subject of the invention is also the use of the compounds as defined above, for transferring polynucleotides (and more generally polyanions) into

cells *in vitro*, *in vivo* or *ex vivo*. More precisely, a subject of the present invention is the use of the compounds as defined above, for preparing a medicinal product intended for treating diseases, in particular diseases which result from a deficiency in a protein or nucleic acid product. The polynucleotide contained in said medicinal product encodes said protein or nucleic acid product, or constitutes said nucleic acid product, which is capable of correcting said diseases *in vivo* or *ex vivo*.

For uses *in vivo*, for example in therapy or for studying gene regulation or creating animal models of pathological conditions, the compositions according to the invention can be formulated for administration via the topical, cutaneous, oral, rectal, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, intratracheal, intraperitoneal, etc. route. Preferably, the compositions of the invention contain a vehicle which is pharmaceutically acceptable for an injectable formulation, in particular for direct injection into the desired organ, or for administration via the topical route (on the skin and/or mucous membrane). They may in particular be isotonic sterile solutions or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow injectable solutes to be constituted. The nucleic acid doses used

for the injection, and also the number of administrations, can be adjusted as a function of various parameters, and in particular as a function of the mode of administration used, of the pathological condition in question, of the gene to be expressed or of the desired duration of treatment. With regard more particularly to the mode of administration, it may be either a direct injection into the tissues, for example into tumors, or into the circulatory system, or it may involve treatment of cells in culture followed by their reimplantation *in vivo*, by injection or transplantation. The relevant tissues in the context of the present invention are, for example, the muscles, skin, brain, lungs, liver, spleen, bone marrow, thymus, heart, lymph, blood, bones, cartilages, pancreas, kidneys, bladder, stomach, intestines, testicles, ovaries, rectum, nervous system, eyes, glands, connective tissues, etc.

Another subject of the present invention relates to a method for treating the human or animal body, comprising the following steps:

(1) bringing the nucleic acid into contact with a transfer agent as defined above, so as to form a complex, and

(2) bringing the cells of the human or animal body into contact with the complex formed in (1).

The invention also relates to a method for transferring nucleic acids into cells, comprising the

following steps:

(1) bringing the nucleic acid into contact with a transfer agent as defined above, so as to form a complex, and

(2) bringing the cells into contact with complex formed in (1).

The cells can be brought into contact with the complex by incubating the cells with said complex (for uses *in vitro* or *ex vivo*), or by injecting the complex into an organism (for uses *in vivo*). The incubation is preferably carried out in the presence of, for example, from 0.01 to 1 000 µg of nucleic acid per 10^6 cells. For administration *in vivo*, nucleic acid doses ranging from 0.01 to 10 mg can, for example, be used.

When the compositions of the invention also contain one or more adjuvants as defined above, the adjuvant(s) is (are) mixed beforehand with the transfer agent according to the invention and/or with the nucleic acid.

The present invention thus provides a particularly advantageous method for transferring nucleic acids *in vivo*, in particular for treating diseases, comprising the *in vivo* or *in vitro* administration of a nucleic acid encoding a protein, or possibly being transcribed into a nucleic acid, which is capable of correcting said disease, said nucleic acid being associated with a compound of general

formula (I) under the conditions defined above.

The agents for transferring nucleic acids, of the invention, can be used in particular for transferring nucleic acids into primary cells or into established lines. They may be fibroblast cells, muscle cells, nerve cells (neurons, astrocytes, glial cells), hepatic cells, hematopoietic cells (lymphocytes, CD34, dendritic cells, etc.), epithelial cells, etc., in differentiated form or pluripotent form (precursors).

Besides the preceding arrangements, the present invention also comprises other characteristics and advantages which will emerge from the examples and figures which follow and which should be considered as illustrating the invention without limiting the scope thereof. In particular, the Applicant provides, with no limitation being implied, various operating protocols and also reaction intermediates which can be used for preparing the transfer agents of general formula (I). Of course, it is within the capabilities of those skilled in the art to use these protocols or intermediate products as a basis for developing similar processes with a view to producing these same compounds. It is also for those skilled in the art to use the synthetic processes described in the various patent applications mentioned above, as a basis for synthesizing the polycation R included in general formula (I) (WO 96/17823, WO 97/18185, WO 97/31935, etc.).

FIGURES

Figure 1: Schematic representation of the plasmid pXL2774 used in the experiments for transferring DNA into cells.

Figure 2: Gene transfer activity *in vitro* in HeLa cells of complexes formed from compound 2 according to the invention without co-lipid, or in the presence of cholesterol and in the presence of DOPE as co-lipids. The y-axis represents luciferase expression in pg/well. The x-axis indicates the transfecting agent/DNA ratio in nmol/µg of DNA.

Figure 3: Gene transfer activity *in vivo* after direct injection into the mouse anterior tibialis muscle of complexes formed from compound 2 according to the present invention in the presence of DOPE (1:1). The y-axis indicates luciferase expression in pg/muscle. The x-axis indicates the compound 2/DNA ratio in nmol/µg of DNA.

EXAMPLES

A \ MATERIALS AND METHODS

a) Materials

- The starting polyamines, such as spermidine, spermine, tris-(2-aminoethyl)amine, phenylenediamine, diaminoalkanes, etc., are commercially available or they can be synthesized using conventional methods (for example by cyanoethylation of commercially available amines so as to obtain branched amines)

- many compounds, such as for example triethylamine, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), benzyl chloroformate, 11-bromoundecanol, etc., are also commercial products.
- Amberlite IR 120 is a commerical ion exchange resin (BDH catalogue).
- Dimethyl sulfoxide (DMSO), treated beforehand with potassium hydroxide, was distilled over calcium hydride and then stored on a 4 Å molecular sieve.
- Dichloromethane was distilled over phosphorus pentoxide and then stored on a 4 Å molecular sieve.
- Tetrahydrofuran (THF) was distilled over sodium in the presence of benzophenone.
- For the reactions requiring anhydrous conditions, all glassware is flame-dried under a nitrogen stream.

b) Methods

- Spectroscopic analyses

The nuclear magnetic resonance (NMR) spectra were recorded on a Brucker MSL 30 spectrometer at a frequency of 300 MHz for the proton and 75 MHz for the carbon. All the chemical shifts are reported in ppm, either relative to the frequency of tetramethylsilane (TMS) or relative to the solvent. The spectra were recorded using either TMS or the residual signal of the solvent as an internal control. The multiplicity of the signals is designated using the following abbreviations: s (singlet), d (doublet), t (triplet),

q (quadruplet) and m (multiplet).

- Chromatography techniques

- The kinetics of the reactions was monitored by thin-layer chromatography (TLC) with a silica gel containing a fluorescent indicator (Merck Silicagel 60 F254) as a support. The chromatograms were developed by spraying an alcoholic solution of anisaldehyde.
- All the column chromatographies were carried out under compressed air pressure with silicagel 60 as the stationary phase (0.05-0.02 mm). The mobile phase used differs depending on the type of synthesis (medium pressure chromatography).
- The HPLC (High Performance Liquid Chromatography) analyses were carried out on a Waters LC 4000 apparatus equipped with a C4-type analytical column sold by Applied Biosystem (stainless steel "Brownlee Columns" 3 cm in length and 0.46 cm in diameter) and a "Waters 486" detector at 220 nm. The stationary phase is 7 micron butyl aquapore, and the mobile phases are demineralized water (2500 cm^3) or acetonitrile (2 500 cm^3) supplemented with trifluoroacetic acid (2.5 cm^3). The flow rate is 1 ml per minute.

B \ SYNTHESIS OF THE TRANSFECTION AGENTS

Example 1: Synthesis of (3-[4-(3-aminopropyl-amino)butylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl α-D-mannopyrannoside (Compound 1)

a) Synthesis of 3-[4-(3-tert-butoxycarbonylaminopropyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonyl-amino]acetic acid (FRM 375)

Sodium cyanoborohydride $NaBH_3CN$ (0.548 g; 8.74 mmol) is added to a solution of spermine (5 g; 24.96 mmol) in methanol (125 ml). The solution is then subjected to vigorous stirring. A solution of glyoxylic acid (2.34 g; 25.46 mmol) in methanol (80 ml) is added over 100 minutes using a pressure-equalizing dropping funnel. After one night, triethylamine (3.86 ml; 27.71 mmol) and di-tert-butyl dicarbonate (27.67 g; 129.79 mmol) solubilized in tetrahydrofuran (55 ml) are added to the mixture. After one night, the mixture is concentrated in a rotary evaporator and then taken up with ethyl acetate (63 ml), then washing is carried out with potassium hydrogen sulfate and brine. Next, the mixture is dried over magnesium sulfate and concentrated. The product obtained is purified by chromatography (CH_2Cl_2/MeOH 9:1). The yield is 30%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.42 (s, 36H, $C(CH_3)_3$), 1.45 (m, 4H, CH_2), 1.60 (m, 4H, CH_2), 3.04-3.33 (m, 12H, CH_2), 3.91 (s, 2H, NCH_2COO).

b) Synthesis of methyl 15-hydroxypentadecanoate

6.66 cm^3 of 2N sodium methylate (13.31 mmol) are added to 10 g of pentadecalactone (41.60 mmol) in 41.60 cm^3 of methanol, at 0°C.

After 9 hours, 9.24 cm^3 of acetic acid are added and allowed to react for 15 minutes. The solution

is then evaporated to dryness under vacuum, the residue is then taken up with dichloromethane and the mixture is washed with sodium bicarbonate. The organic phase obtained is dried using magnesium sulfate and the solvent is evaporated in a rotary evaporator. The purification is carried out in a 6:4 hexane/ethyl acetate mixture. Methyl 1-ol-pentadecanoate is obtained with a yield of 80%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.26 (m, 12H, $(CH_2)_{10}$), 1.5-1.6 (m, 4H, H-2 and H-13), 2.30 (t, 2H, J=7.60 Hz, H-14), 3.64 (t, 1H, *J= 5.84* Hz, H-1), 3.67 (s, 3H, H-16).

c) Synthesis of methyl pentadecanoate 2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside

5.26 cm^3 of tin chloride (44.94 mmol) are added to 8.72 g of pentaacetylated mannose (22.47 mmol) in 56 cm^3 of dichloromethane over 30 minutes, at 0°C. 7.34 g of methyl 1-ol-pentadecanoate previously obtained in a) (26.96 mmol) are then added. After 2 hours, the reaction mixture is diluted with ethyl ether and poured into a solution of sodium hydrogen phosphate ($NaHPO_4$). The aqueous phases are extracted with diethyl ether and the organic phases are successively washed with a solution of sodium carbonate, and brine, and then dried over magnesium sulfate. The product obtained after evaporation to dryness under vacuum is purified by medium pressure chromatography in a 7:3 heptane/ethyl acetate mixture. The yield is 53%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.26 (m, 20H, $(CH_2)_{10}$), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 2.01, 2.05, 2.12 and 2.17 (s, 3H, $OCO\underline{CH_3}$), 2.29 (t, 2H, *J= 7.62* Hz, H-14), 3.40 (m, 1H, *J= 7.89* Hz, $O\underline{CH_a}CH_2$), 3.66 (m, 1H, *J= 7.89* Hz, $O\underline{CH_b}CH_2$), 3.67 (s, 3H, $COO\underline{CH_3}$), 4.05 (ddd, 1H, *J= 9.56* Hz and 5.57 Hz, H-5), 4.1 (dd, 1H, *J= 5.57* Hz and *12.32* Hz, H-6a), 4.29 (dd, 1H, *J= 5.57* Hz and *12.32* Hz, H-6b), 4.8 (d, 1H, *J= 1.85* Hz, H-1), 5.23 (dd, 1H, *J= 1.85* Hz and 3.23 Hz, H-2), 5.27 (dd, 1H, *J= 9.97* Hz and *9.56* Hz, H-4), 5.35 (dd, 1H, *J= 9.97* Hz and *3.23* Hz, H-3).

d) Synthesis of methyl pentadecanoate α-D-mannopyranoside

3.63 g of product obtained in the preceding step (6.01 mmol) dissolved in 12 cm^3 of methanol are treated with 3 cm^3 of 2N sodium methylate (6.01 mmol). When the reaction is complete, the latter is neutralized with Amberlite IR120 (1 weight equivalent/volume), filtered and evaporated to dryness under vacuum.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.28 (m, 20H, $(CH_2)_{10}$), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 2.34 (t, 2H, *J= 7.62* Hz, H-14), 3.41 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.74 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.67 (s, 3H, $\underline{CH_3}OCO$), 3.5-3.82 (m, 6H, H-2, H-3, H-4, H-5 and H-6), 4.75 (d, 1H *J= 1.82* Hz, H-1).

e) Synthesis of methyl pentadecanoate 2,3,4,6-tetra-O-benzyl-α-D-mannopyranoside

4.54 g of potassium iodide (27.36 mmol), 1.09 g of 60% sodium hydride (27.36 mmol) and 3.25 cm^3 of benzyl bromide (27.36 mmol) are added successively to 2 g (4.56 mmol) of product obtained in the preceding step d) dissolved in 20 cm^3 of dimethylformamide (DMF). After 12 hours, 18.24 cm^3 of a saturated ammonium chloride solution are added and allowed to react for 10 minutes. The mixture is then diluted with water and the organic phase is extracted with ethyl acetate. This organic phase is then washed with water and brine, and is finally dried with magnesium sulfate. An additional wash is, moreover, carried out using a saturated sodium thiosulfate solution in order to remove the iodide ions. The mixture is evaporated under vacuum and the resulting oil is purified in a 9:1 heptane/ethyl acetate mixture. The product is obtained with a yield of 60%.

^{1}H NMR ($CDCl_3$): d (ppm) 1.28 (m, 20H, $(CH_2)_{10}$), 1.49 (m, 2H, $OCH_2\underline{CH_2}$), 1.59 (m, 2H, H-13), 2.31 (t, 2H, *J= 7.62* Hz, H-14), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.67 (s, 3H, $\underline{CH_3}OCO$), 3.75 (m, 1H, *J= 8.97* Hz and 6.21 Hz, H-5), 3.78 (s, 2H, $\underline{CH_2}$Phe), 3.90 (dd, 1H *J*= 6.21 Hz and *J*= 11.82 Hz, H-6a), 3.97 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6b), 4.07 (s, 2H, $\underline{CH_2}$Phe), 4.52 (dd, *J*= 2.91 Hz and 7.83 Hz, H-3), 4.57 (s, 2H, $\underline{CH_2}$Phe), 4.63 (s, 2H, $\underline{CH_2}$Phe), 4.69 (dd, 1H, *J*= 2.52 Hz and 2.91 Hz, H-2), 4.74 (1H, *J*= 2.52 Hz, H-1), 4.85 (dd, 1H, *J*= 7.83 Hz and 8.97 Hz, H-4), 7.35

(m, 20H, Phe).

f) Synthesis of pentadecanoic acid 2,3,4,6-tetra-O-benzyl-α-D-mannopyranoside

4.68 cm^3 of a 25% sodium hydroxide solution are added to 0.50 g (0.73 mmol) of product obtained in the preceding step e) dissolved in 7 cm^3 of methanol. The reaction mixture is heated under reflux for 30 minutes. The mixture is then neutralized at low temperature with a 5% hydrochloric acid solution. The organic phase is extracted with ethyl acetate and evaporated to dryness under vacuum. The purification is carried out in a 4:6 heptane/ethyl acetate mixture. The product is obtained with a yield of 62%.

^{1}H NMR ($CDCl_3$): d (ppm) 1.28 (m, 20H, $(CH_2)_{10}$), 1.49 (m, 2H, $OCH_2\underline{CH_2}$), 1.59 (m, 2H, H-13), 2.34 (t, 2H, *J= 7.62* Hz, H-14), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.75 (m, 1H, *J*= 8.97 Hz and 6.21 Hz, H-5), 3.78 (s, 2H, $\underline{CH_2}$Phe), 3.90 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6a), 3.97 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6b), 4.07 (s, 2H, $\underline{CH_2}$Phe), 4.52 (dd, *J*= 2.91 Hz and 7.83 Hz, H-3), 4.57 (s, 2H, $\underline{CH_2}$Phe), 4.63 (s, 2H, $\underline{CH_2}$Phe), 4.69 (dd, 1H, *J*= 2.52 Hz and 2.91 Hz, H-2), 4.74 (1H, *J*= 2.52 Hz, H-1), 4.85 (dd, 1H, *J*= 7.83 Hz and 8.97 Hz, H-4), 7.35 (m, 20H, Phe).

g) Synthesis of N-octadecyl-15-carbamoylpentadecanyl 2,3,4,6-tetra-O-benzyl-α-D-mannopyranoside

0.23 g of BOP (0.52 mmol), 0.21 cm^3 of

diisopropylethylamine (1.48 mmol) and 0.12 g of octadecylamine (0.44 mmol) are added successively to 0.29 g (0.37 mmol) of a solution of product obtained in the preceding step f) dissolved in 5 cm^3 of chloroform. When the reaction is complete, the mixture is diluted with dichloromethane and washed with water. It is then dried using magnesium sulfate and evaporated to dryness under vacuum. The product obtained is purified by medium pressure chromatography in a 6:4 heptane/ethyl acetate mixture. The product is obtained with a yield of 98%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.47 (m, 4H, $OCH_2\underline{CH_2}$ and H-17), 1.58 (m, 2H, H-13), 2.13 (t, 2H, *J= 7.92* Hz, H-14), 3.23 (m, 2H, H-16), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.75 (m, 1H, *J= 8.97* Hz and 6.21 Hz, H-5), 3.78 (s, 2H, $\underline{CH_2}$Phe), 3.90 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6a), 3.97 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6b), 4.07 (s, 2H, $\underline{CH_2}$Phe), 4.52 (dd, *J*= 2.91 Hz and 7.83 Hz, H-3), 4.57 (s, 2H, $\underline{CH_2}$Phe), 4.63 (s, 2H, $\underline{CH_2}$Phe), 4.69 (dd, 1H, *J*= 2.52 Hz and 2.91 Hz, H-2), 4.74 (1H, *J*= 2.52 Hz, H-1), 4.85 (dd, 1H, *J*= 7.83 Hz and 8.97 Hz, H-4), 5.37 (band, 1H, $\underline{HN}CO$), 7.35 (m, 20H, Phe).

h) Synthesis of 15-octadecylaminopentadecanyl 2,3,4,6-tetra-O-benzyl-α-D-mannopyranoside

0.056 g of lithium aluminum hydride $AlLiH_4$ (1.50 mmol) is added to 0.77 g (0.75 mmol) of product

obtained in the preceding step g) in 15 cm^3 of anhydrous tetrahydrofuran (THF). The mixture is heated under reflux for 10 hours. The reaction mixture is then cooled in an ice bath and 56 µl of water are added, followed by 112 µl of 2N sodium hydroxide after 10 minutes and, finally, a further 56 µl of water 10 minutes later. The mixture is filtered and evaporated to dryness under vacuum. The product obtained is purified in a 9:2:0.5 dichloromethane/methanol/28% ammonia mixture. The product is obtained with a yield of 86%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.4-1.6 (m, 9H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17 and NH), 2.57 (t, 4H, *J*= 7.92 Hz, H-15 and H-16), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.75 (m, 1H, *J= 8.97* Hz and 6.21 Hz, H-5), 3.78 (s, 2H, $\underline{CH_2}$Phe), 3.90 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6a), 3.97 (dd, 1H, *J*= 6.21 Hz and *J*= 11.82 Hz, H-6b), 4.07 (s, 2H, $\underline{CH_2}$Phe), 4.52 (dd, *J*= 2.91 Hz and 7.83 Hz, H-3), 4.57 (s, 2H, $\underline{CH_2}$Phe), 4.63 (s, 2H, $\underline{CH_2}$Phe), 4.69 (dd, 1H, *J*= 2.52 Hz and 2.91 Hz, H-2), 4.74 (1H, *J*= 2.52 Hz, H-1), 4.85 (dd, 1H, *J*= 7.83 Hz and 8.97 Hz, H-4), 7.35 (m, 20H, Phe).

i) Synthesis of (3-[4-(3-tert-butoxycarbonylamino-propyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl 2,3,4,6-tetra-O-benzyl-α-D-

mannopyranoside

0.38 g of BOP (0.85 mmol), 0.425 cm^3 of diisopropylethylamine (2.44 mmol) and 0.48 g of 3-[4-(3-tert-butoxycarbonylaminopropyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonylamino]-acetic acid (FRM 375) (0.73 mmol) obtained in step a) are added successively to 0.63 g (0.61 mmol) of a solution of product obtained previously in step h) in 10 cm^3 of chloroform. After 4 hours, the mixture is diluted with dichloromethane and washed with water. It is dried using magnesium sulfate and evaporated to dryness under vacuum. The product obtained is purified by medium pressure chromatography in a 6:4 heptane/ethyl acetate mixture. The product is obtained with a yield of 80%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 1.46 (m, 36H, Boc), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 3.09-3.33 (m, 12H, H-36, H-38, H-39, H-42, H-43 and H-45), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.75 (m, 1H, *J=* 8.97 Hz and 6.21 Hz, H-5), 3.78 (s, 2H, $\underline{CH_2}$Phe), 3.90 (dd, 1H, *J=* 6.21 Hz and *J=* 11.82 Hz, H-6a), 3.97 (dd, 1H, *J=* 6.21 Hz and *J=* 11.82 Hz, H-6b), 4.07 (s, 2H, $\underline{CH_2}$Phe), 4.52 (dd, *J=* 2.91 Hz and 7.83 Hz, H-3), 4.57 (s, 2H, $\underline{CH_2}$Phe), 4.63 (s, 2H, $\underline{CH_2}$Phe), 4.69 (dd, 1H, *J=* 2.52 Hz and 2.91 Hz, H-2), 4.74 (1H, *J=* 2.52 Hz, H-1), 4.85 (dd, 1H,

J= 7.83 Hz and 8.97 Hz, H-4), 7.35 (m, 18H, Phe).

j) Synthesis of (3-[4-(3-tert-butoxycarbonylamino-propyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl α-D-mannopyranoside

10% palladium on charcoal (0.027 g) is added to 0.63 g (0.38 mmol) of product obtained in the preceding step i) in 5 cm^3 of methanol. The solution is stirred under hydrogen pressure at room temperature. After 6 hours, it is filtered and then evaporated to dryness under vacuum. The reaction is quantitative.

^{1}H NMR (CD_3OD): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 1.46 (m, 36H, Boc), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 3.09-3.33 (m, 12H, H-36, H-38, H-39, H-42, H-43 and H-45), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.5-3.82 (m, 6H, H-2, H-3, H-4, H-5 and H-6), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 4.72 (1H, *J*= 2.52 Hz, H-1).

k) Synthesis of (3-[4-(3-aminopropylamino)butylamino]-methylenecarbamoyl)-15-pentadecanyl-16-octadecyl α-D-mannopyranoside (compound 1)

21.50 cm^3 of distilled tetrahydrofuran (TFA) are added to 0.37 g (0.28 mmol) of product obtained in the preceding step j). After 1 hour, the reaction mixture is concentrated at low temperature and freeze-dried. The degree of purity of the product dissolved in methanol is verified by HPLC as described in the

"Materials and Methods" section.

^{1}H NMR (CD_3OD): 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 14H, $(CH_2)_{25}$), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 2.92 (m, 2H, H-45), 2.92-3.17 (m, 12H, H-36, H-38, H-39, H-42, H-43), 3.34 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.5-3.82 (m, 6H, H-2, H-3, H-4, H-5 and H-6), 3.63 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 4.72 (1H, *J= 2.02* Hz, H-1).

Example 2: Synthesis of (3-[4-(3-aminopropylamino)-butylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl 6-deoxy-α-L-mannopyranoside (compound 2)

a) Synthesis of 3-[4-(3-tert-butoxycarbonylaminopropyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonyl-amino]acetic acid (FRM 375)

Sodium cyanoborohydride $NaBH_3CN$ (0.548 g; 8.74 mmol) is added to a solution of spermine (5 g; 24.96 mmol) in methanol (125 ml). The solution is then subjected to vigorous stirring. A solution of glyoxylic acid (2.34 g; 25.46 mmol) in methanol (80 ml) is added over 100 minutes using a pressure-equalizing dropping funnel. After one night, triethylamine (3.86 ml; 27.71 mmol) and di-tert-butyl dicarbonate (27.67 g; 129.79 mmol) solubilized in tetrahydrofuran (55 ml) are added to the mixture. After one night, the mixture is concentrated in a rotary evaporator and then taken up with ethyl acetate (63 ml), then washing is carried out with potassium hydrogen sulfate and brine. Next, the

mixture is dried over magnesium sulfate and concentrated. The product obtained is purified by chromatography (CH_2Cl_2/MeOH 9:1). The yield is 30%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.42 (s, 36H, $C(CH_3)_3$), 1.45 (m, 4H, CH_2), 1.60 (m, 4H, CH_2), 3.04-3.33 (m, 12H, CH_2), 3.91 (s, 2H, NCH_2COO).

b) Synthesis of methyl 15-hydroxypentadecanoate

6.66 cm^3 of 2N sodium methylate (13.31 mmol) are added to 10 g of pentadecalactone (41.60 mmol) in 41.60 cm^3 of methanol, at 0°C. After 9 hours, 9.24 cm^3 of acetic acid are added and allowed to react for 15 minutes. The solution is then evaporated to dryness under vacuum, the residue is then taken up with dichloromethane and the mixture is washed with sodium bicarbonate. The organic phase obtained is dried using magnesium sulfate and the solvent is evaporated in a rotary evaporator. The purification is carried out in a 6:4 hexane/ethyl acetate mixture. Methyl 1-ol-pentadecanoate is obtained with a yield of 80%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.26 (m, 12H, $(CH_2)_{10}$), 1.5-1.6 (m, 4H, H-2 and H-13), 2.30 (t, 2H, *J*= *7.60* Hz, H-14), 3.64 (t, 1H, *J*= *5.84* Hz, H-1), 3.67 (s, 3H, H-16).

c) Synthesis of methyl pentadecanoate 2,3,4-tri-O-acetyl-6-deoxy-α-L-mannopyranoside

2.49 cm^3 of tin chloride (21.30 mmol) are added to 3.55 g of tetracetylated rhamnose (10.65 mmol) in 27 cm^3 of dichloromethane over 30 minutes, at 0°C. 3.48 g of methyl 1-ol-pentadecanoate previously

obtained (12.78 mmol) are then added. After 2 hours, the reaction mixture is diluted with ethyl ether and poured into a solution of sodium phosphate (Na_2PO_4). The aqueous phases are extracted with diethyl ether and the organic phases are successively washed with a sodium carbonate solution, and brine, and then dried using magnesium sulfate. After evaporation to dryness under vacuum, the product is purified by medium pressure chromatography in a 7:3 heptane/ethyl acetate mixture. The product is obtained with a yield of 60%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.20 (d, 3H, *J= 6.45* Hz, H-6), 1.26 (m, 20H, $(CH_2)_{10}$), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 1.98, 2.04 and 2.16 (s, 3H, $OCO\underline{CH_3}$), 2.29 (t, 2H, *J= 7.62* Hz, H-14), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.67 (s, 3H, $COO\underline{CH_3}$), 3.88 (m, 1H, *J= 6.45* Hz and *9.97* Hz, H-5), 4.70 (d, 1H, *J= 1.72* Hz, H-1), 5.06 (dd, 1H, *J= 9.97* Hz and *9.97* Hz, H-4), 5.22 (dd, 1H, *J= 1.72* Hz and *3.52* Hz, H-2), 5.30 (dd, 1H, *J= 3.52* Hz and *9.97* Hz, H-3).

d) Synthesis of methyl pentadecanoate α-deoxy-L-6-mannopyranoside

5.08 g of product obtained in step c) (9.34 mmol) dissolved in 20 cm^3 of methanol are treated with 9.34 ml of 2N sodium methylate (18.68 mmol). When the reaction is complete, the reaction mixture is neutralized with Amberlite IR120, filtered and evaporated to dryness under vacuum.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.20 (d, 3H, *J= 6.45* Hz, H-6),

1.26 (m, 20H, $(CH_2)_{10}$), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 2.29 (t, 2H, *J= 7.62* Hz, H-14), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.67 (s, 3H, $C\underline{H_3}OCO$), 3.6-3.9 (m, 4H, H-2, H-3, H-4 and H-5), 4.70 (d, 1H, *J= 1.72* Hz, H-1).

e) Synthesis of methyl pentadecanoate 2,3,4-tri-O-benzyl-6-deoxy-α-L-mannopyranoside

3.32 g of potassium iodide (20.00 mmol), 0.80 g of 60% sodium hydride (20.00 mmol) and 2.38 cm^3 of benzyl bromide (20.00 mmol) are successively added to 2.09 g (5.00 mmol) of product obtained in the preceding step d) in 30 cm^3 of anhydrous dimethylformamide (DMF). After 12 hours, 20 cm^3 of a saturated ammonium chloride solution are added and allowed to react for 10 minutes. The mixture is then diluted with water and the organic phase is extracted with ethyl acetate. This organic phase is then washed with water and brine, and is finally dried with magnesium sulfate.

An additional wash is, moreover, carried out using a saturated sodium thiosulfate solution in order to remove the iodide ions. The mixture is evaporated to dryness under vacuum and the resulting oil is purified in a 9:1 heptane/ethyl acetate mixture. The product is obtained with a yield of 60%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.28 (m, 20H, $(CH_2)_{10}$), 1.33 (d, 3H, *J= 6.21* Hz, H-6), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 2.31 (t, 2H, *J= 7.62* Hz, H-14), 3.40 (m, 1H,

J= *6.71* Hz, O$\underline{CH_a}CH_2$), 3.61 (dd, 1H, *J*= *8.96* Hz and *9.5* Hz, H-4), 3.66 (m, 1H, *J*= *6.71* Hz, O$\underline{CH_b}CH_2$), 3.67 (s, 3H, $\underline{CH_3}OCO$), 3.68 (m, 1H, *J*= *9.5* Hz and *6.21* Hz, H-5), 3.75 (dd, 1H, *J*= *2.01* Hz and *3.02* Hz, H-2), 3.88 (dd, *J*= *3.02* Hz and *8.96* Hz, H-3), 4.64 (s, 6H, $\underline{CH_2}$Phe), 4.73 (1H, *J*= *2.01* Hz, H-1), 7.35 (m, 15H, Phe).

f) Synthesis of pentadecanoic acid 2,3,4-tri-O-benzyl-6-deoxy-α-L-mannopyranoside

4.68 ml of 25% sodium hydroxide are added to 0.50 g (0.73 mmol) of a solution of product obtained in the preceding step e) in 7 cm^3 of methanol. The reaction mixture is heated under reflux for 30 minutes. The mixture is then neutralized at low temperature with a 5% hydrochloric acid solution. The organic phase is extracted with ethyl acetate and evaporated to dryness under vacuum. The purification is carried out in a 4:6 heptane/ethyl acetate mixture. The product is obtained with a yield of 72%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.28 (m, 20H, $(CH_2)_{10}$), 1.33 (d, 3H, *J*= 6.21 Hz, H-6), 1.59 (m, 4H, $OCH_2\underline{CH_2}$ and H-13), 2.34 (t, 2H, *J*= *7.62* Hz, H-14), 3.40 (m, 1H, *J*= *6.71* Hz, O$\underline{CH_a}CH_2$), 3.61 (dd, 1H, *J*= *8.96* Hz and *9.5* Hz, H-4), 3.66 (m, 1H, *J*= *6.71* Hz, O$\underline{CH_b}CH_2$), 3.68 (m, 1H, *J*= *9.5* Hz and *6.21* Hz, H-5), 3.75 (dd, 1H, *J*= *2.01* Hz and *3.02* Hz, H-2), 3.88 (dd, *J*= *3.02* Hz and *8.96* Hz, H-3), 4.64 (s, 6H, $\underline{CH_2}$Phe), 4.73 (1H, *J*= *2.01* Hz, H-1), 7.35 (m, 20H, Phe).

g) Synthesis of N-octadecyl-15-carbamoylpentadecanyl

2,3,4-tri-O-benzyl-6-deoxy-α-L-mannopyranoside

0.69 g of BOP (1.56 mmol), 0.72 cm^3 of diisopropylethylamine (4.16 mmol) and 0.34 g of octadecylamine (1.25 mmol) are added successively to 0.70 g (1.04 mmol) of a solution of product previously obtained in step f) in 13 cm^3 of chloroform. When the reaction is complete, the mixture is diluted with dichloromethane, washed with water, dried over magnesium sulfate and evaporated to dryness under vacuum. The product obtained is purified by medium pressure chromatography in a 6:4 heptane/ethyl acetate mixture. The product is obtained with a yield of 84%.

^{1}H NMR ($CDCl_3$): δ (ppm 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.33 (d, 3H, *J*= 6.21 Hz, H-6), 1.47 (m, 4H, $OCH_2\underline{CH_2}$ and H-17), 1.58 (m, 2H, H-13), 2.13 (t, 2H, *J= 7.92* Hz, H-14), 3.23 (m, 2H, H-16), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.61 (dd, 1H, *J= 8.96* Hz and *9.5* Hz, H-4), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.68 (m, 1H, *J*= 9.5 Hz and 6.21 Hz, H-5), 3.75 (dd, 1H, *J= 2.01* Hz and *3.02* Hz, H-2), 3.88 (dd, *J= 3.02* Hz and *8.96* Hz, H-3), 4.64 (s, 6H, $\underline{CH_2}$Phe), 4.73 (1H, *J*= 2.01 Hz, H-1), 5.37 (band, 1H, HNCO), 7.35 (m, 15H, Phe).

h) Synthesis of 15-octadecylaminopentadecanyl 2,3,4-tri-O-benzyl-6-deoxy-α-L-mannopyranoside

0.065 g of lithium aluminum hydride $AlLiH_4$ (1.72 mmol) is added to 0.81 g (0.86 mmol) of product obtained in the preceding step g) in 15 cm^3 of anhydrous

tetrahydrofuran (THF), and the mixture is heated under reflux for 10 hours. The reaction mixture is then cooled in an ice bath and 65 µl of water are added, followed by 130 µl of 2N sodium hydroxide after 10 minutes and, finally, a further 65 µl of water after 10 minutes. The mixture is filtered and evaporated to dryness under vacuum. The purification is carried out in a 9:2:0.5 dichloromethane/methanol/28% ammonia mixture. The product is obtained with a yield of 93%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.33 (d, 3H, *J*= 6.21 Hz, H-6), 1.4-1.6 (m, 9H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17 and NH), 2.57 (t, 4H, *J= 7.92* Hz, H-15 and H-16), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.61 (dd, 1H, *J= 8.96* Hz and *9.5* Hz, H-4), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.68 (m, 1H, *J= 9.5* Hz and 6.21 Hz, H-5), 3.75 (dd, 1H, *J= 2.01* Hz and *3.02* Hz, H-2), 3.88 (dd, *J= 3.02* Hz and *8.96* Hz, H-3), 4.64 (s, 6H, $\underline{CH_2}$Phe), 4.73 (1H, *J*= 2.01 Hz, H-1), 7.35 (m, 15H, Phe).

i) Synthesis of (3-[4-(3-tert-butoxycarbonylamino-propyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl 2,3,4-tri-O-benzyl-6-deoxy-α-L-mannopyranoside

0.53 g of BOP (1.20 mmol), 0.30 cm^3 of diisopropylethylamine (1.72 mmol) and 0.62 g of FRM 375 obtained in step a) (0.95 mmol) are added successively to 0.78 g (0.86 mmol) of a solution of product obtained

in the preceding step h) dissolved in 7 cm^3 of chloroform. After 4 hours, the mixture is diluted with dichloromethane, washed with water, dried over magnesium sulfate and evaporated to dryness under vacuum. The product obtained is purified by "flash" chromatography in a 6:4 heptane/ethyl acetate mixture. The product is obtained with yield of 72%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.27 (m, 50H, $(CH_2)_{25}$), 1.33 (d, 3H, *J*= 6.21 Hz, H-6), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 1.46 (m, 36H, Boc), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 3.09-3.33 (m, 12H, H-36, H-38, H-39, H-42, H-43 and H-45), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.65 (s, 2H, $\underline{CH_2}$Phe), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.68 (m, 1H, *J*= 9.5 Hz and 6.21 Hz, H-5), 3.99 (s, 2H, $\underline{CH_2}$Phe), 4.02 (dd, 1H, *J*= 8.96 and 9.5 Hz, H-4), 4.32 (s, 2H, $\underline{CH_2}$Phe), 4.57 (dd, 1H, *J*= 2.01 Hz and 3.02 Hz, H-2), 4.73 (1H, *J*= 2.01 Hz, H-1), 4.82 (dd, *J*= 3.02 Hz and 8.96 Hz, H-3), 7.35 (m, 18H, Phe).

j) Synthesis of (3-[4-(3-tert-butoxycarbonylamino-propyl-tert-butoxycarbonylamino)butyl-tert-butoxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecyl 6-deoxy-α-L-mannopyranoside

10% (0.034 g) palladium on charcoal is added to 0.74 g (0.48 mmol) of product obtained in the preceding step i) dissolved in 10 cm^3 of methanol. The solution is stirred under hydrogen pressure at room temperature. After 4 hours, it is filtered and then

evaporated to dryness under vacuum. The reaction is quantitative.

^{1}H NMR (CD_3OD): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.20 (d, 3H, *J= 6.45* Hz, H-6), 1.27 (m, 14H, $(CH_2)_{25}$), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 1.46 (m, 36H, Boc), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 3.09-3.33 (m, 12H, H-36, H-38, H-39, H-42, H-43 and H-45), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.6-3.9 (m, 4H, H-2, H-3, H-4 and H-5), 4.73 (1H, *J*= 2.01 Hz, H-1).

k) Synthesis of (3-[4-(3-aminopropylamino)butylamino]-methylenecarbamoyl)-15-pentadecanyl-16-octadecyl 6-deoxy-α-L-mannopyranoside (compound 2)

24 cm^3 of distilled tetrahydrofuran (TFA) are added to 0.40 g (0.31 mmol) of product obtained in the preceding step j). After 1 hour, the reaction mixture is evaporated to dryness under vacuum at low temperature, and then freeze-dried. The degree of purity of the product dissolved in methanol is verified by HPLC.

^{1}H NMR (CD_3OD): δ (ppm) 0.88 (t, 3H, *J= 6.36* Hz, H-33), 1.20 (d, 3H, *J= 6.45* Hz, H-6'), 1.27 (m, 14H, $(CH_2)_{25}$), 1.4-1.6 (m, 17H, $OCH_2\underline{CH_2}$, H-17, H-14, H-17, H-37, H-40, H-41 and H-44), 2.8-2.9 (m, 6H, H-15, H-16 and H-35), 2.92 (m, 2H, H-45), 2.92-3.17 (m, 12H, H-36, H-38, H-39, H-42, H-43), 3.40 (m, 1H, *J= 6.71* Hz, $O\underline{CH_a}CH_2$), 3.66 (m, 1H, *J= 6.71* Hz, $O\underline{CH_b}CH_2$), 3.6-3.9 (m, 4H, H-2, H-3, H-4 and H-5), 4.73 (1H, *J*= 2.01 Hz, H-1).

Example 3: Synthesis of 1-[-(3-[4-(3-aminopropylamino)-butylaminopropylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecanyl 6-deoxy-β-L-galactopyranoside (compound 3)

a) Synthesis of {3-[4-(3-benzyloxycarbonylaminopropyl-benzyloxycarbonylamino)butylbenzyloxycarbonylamino]-propylamino}acetic acid

Sodium cyanoborohydride $NaBH_3CN$ (1.10 g; 17.47 mmol) is added to a solution of spermine (10 g; 49.91 mmol) in methanol (200 ml). The solution is then subjected to vigorous stirring. A solution of glyoxylic acid (4.59 g; 49.91 mmol) in methanol (120 ml) is added over 100 minutes using a pressure-equalizing dropping funnel. After one night, the reaction mixture is placed in an ice bath, and 2N sodium hydroxide (34 ml) and benzyl chloroformate (14.25 ml; 99.82 mmol) are added successively in 10 portions. Vigorous mixing is then carried out while maintaining the bath at between 5°C and 10°C. After 2 hours at room temperature, the mixture is extracted with ether and neutralized with a 5N hydrochloric acid solution. The organic phase is then dried over magnesium sulfate and concentrated with a rotary evaporator. The product obtained is purified by chromatography (100% CH_2Cl_2, then 9:1 CH_2Cl_2/MeOH). The yield is 52%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.28 (t, 4H, CH_2), 1.60 (m, 4H, CH_2), 3.04-3.33 (m, 12H, CH_2), 3.49 (s, 2H, NCH_2COO), 5.07 (s, 8H, CH_2), 7.27 (m, 20H, Phe).

b) Synthesis of methyl 15-hydroxypentadecanoate

Pentadecalactone (10 g; 41.6 mmol) dissolved in methanol (41.6 ml) is treated with 2N sodium methylate (6.656 ml; 13.31 mmol) at 0°C. After 9 hours, 9.24 ml of acetic acid are added and allowed to react for 15 minutes. The solution is then concentrated and the resulting oil is dissolved in dichloromethane and washed with sodium bicarbonate. After decanting, the organic phase is dried over magnesium sulfate and evaporated. The purification is carried out in a 6:4 hexane/ethyl acetate (AcOEt) mixture so as to give methyl 15-hydroxypentadecanoate with a yield of 80%.

^{1}H NMR ($CDCl_3$): δ (ppm) 1.29 (m, 20H, $(CH_2)_{10}$), 1.5-1.6 (m, 4H, H-2 and H-13), 2.30 (t, 2H, *J=7.60* Hz, H-14), 3.64 (t, 1H, *J= 5.84* Hz, H-1), 3.67 (s, 3H, H-16).

c) Synthesis of N-octadecyl-15-hydroxypentadecanamide

10 g of methyl 15-hydroxypentadecanoate obtained in the preceding step b) (36.85 mmol) and 19.86 g of octadecylamine (73.70 mmol) are melted at 150°C under vacuum. After 24 hours, the mixture is cooled and diluted with dichloromethane. A precipitate is obtained which is filtered through a Büchner funnel. The solid obtained is then recrystallized in methanol so as to give N-octadecyl-15-hydroxypentadecanamide with a yield of 100%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.96* Hz, H-33), 1.26 (m, 54H, $(CH_2)_{27}$), 1.4-1.6 (m, 6H, H-2, H-13 and H-17), 2.30 (t, 2H, *J= 7.60* Hz, H-14), 3.25 (m, 2H,

H-16), 3.64 (t, 2H, *J= 5.84* Hz, H-1), 5.39 (band NHCO).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.48 (C-33), 25.3 and 26.3 (C-2 and C-13), 29.72 ($(CH_2)_{27}$), 36.7 and 34.8 (C-14 and C-16), 63.6 (C-1), 174.31 (CO).

d) Synthesis of 15-octadecylaminopentadecanol

2.98 g of lithium aluminum hydride $LiAlH_4$ (78.44 mmol) are added to a solution of 20 g of N-octadecyl-15-hydroxypentadecanamide obtained in the preceding step c) (39.22 mmol) in anhydrous tetrahydrofuran (250 ml). The reaction is carried out under reflux for 10 hours. After having cooled the reaction mixture, water (2.98 ml) and 2N sodium hydroxide (2.98 ml) are added successively. After 10 minutes, water is added again (2.98 ml). The precipitate formed is filtered through a Büchner funnel and the filtrate is concentrated in a rotary evaporator so as to give 15-octadecylaminopentadecanol.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.96* Hz, H-33), 1.26 (m, 54H, $(CH_2)_{27}$), 1.43-1.59 (m, 7H, H-2, H-14, H-17 and band NH), 1.5-1.6 (m, 4H, H-2 and H-13), 2.60 (t, 4H, *J= 6.50* Hz, H-15 and H-16), 3.64 (t, 2H, *J= 5.84* Hz, H-1).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.48 (C-33), 25.3 and 26.3 (C-2 and C-14), 29.72 ($(CH_2)_{27}$), 51.7 (C-15 and C-16), 63.6 (C-1).

e) Synthesis of N-[benzyloxycarbonyl]-15-octadecyl-aminopentadecanol

7.89 ml of benzyl chloroformate (55.26 mmol)

are added dropwise to a solution cooled to 0°C of 15-octadecylaminopentadecanol obtained in the preceding step d) (13.71 g; 27.63 mmol) and of triethylamine (7.7 ml; 55.26 mmol) in dry dichloromethane (150 ml). After 10 minutes, the pH of the mixture is verified. The reaction mixture is then left at room temperature overnight. The solution is then washed with water, dried over magnesium sulfate ($MgSO_4$) and concentrated. The reaction mixture is purified by chromatography (6:4 heptane/AcOEt). N-[benzyloxycarbonyl]-15-octadecyl-aminopentadecanol is obtained with a yield of 70%.

^{1}H NMR ($CDCl_3$): δ (ppm) 0.88 (t, 3H, *J= 6.96* Hz, H-33), 1.26 (m, 54H, $(CH_2)_{27}$), 1.43-1.59 (m, 6H, H-2, H-14, H-17), 3.20-3.22 (m, 4H, H-15 and H-16), 3.64 (t, 2H, *J= 5.84* Hz, H-1), 5.12 (s, 2H, OCH_2Phe), 7.34 (m, 5H, Phe).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.48 (C-33), 25.8, 26.9 and 31.94 (C-2, C-14 and C-17), 29.72 ($(CH_2)_{27}$)), 47.26-48.04 (C-15 and C-16), 63.08 (C-1), 66.79 (OCH_2), 128.40 (Phe).

f) Synthesis of 15-[N-(benzyloxycarbonyl)octadecyl-amino]pentadecanyl 2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranoside

1.5 g of tetraacetylated fucose (4.52 mmol) are reacted with 0.634 ml of tin tetrachloride (5.42 mmol) in dried acetonitrile (50 ml) for 30 minutes. 3.132 g of N-[benzyloxycarbonyl]-15-octadecylaminopentadecanol obtained in the preceding

step e) (4.97 mmol) are then added. After 5 hours, the reaction is extracted and the product obtained is then purified by chromatography (6:4 heptane/ethyl acetate). The yield is 69%.

^{1}H NMR: δ (ppm) 0.87 (t, 3H, *J= 6.96* Hz, H-33), 1.2 (d, 3H, *J= 6.51* Hz, H-6), 1.25 (m, 54H, $(CH_2)_{27}$), 1.52 (m, 6H, $OCH_2\underline{CH_2}$, H-14 and H-17), 1.95, 2.05 and 2.15 (s, 3H, $OCO\underline{CH_3}$), 3.14-3.25 (m, 4H, H-15 and H-16), 3.44 (m, 1H, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, $O\underline{CH_b}CH_2$), 3.79 (m, 1H, H-5), 4.41 (d, 1H, *J= 7.98* Hz, H-1), 4.99 (dd, 1H, *J= 3.52* Hz and *10.46* Hz, H-3), 5.09 (s, 2H, $O\underline{CH_2}$Phe), 5.16 (dd, 1H, *J= 7.98* Hz and *10.46* Hz, H-2), 5.23 (dd, *J= 3.52* Hz and *3.31* Hz, H-4), 7.32 (m, 5H, Phe).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.68 (C-33), 17.31 (C-2), 20.75 ($\underline{CH_3}COO$), 27.29 (C-6), 29.72 ($(CH_2)_{27}$)), 25.89-31.98 ($OCH_2\underline{CH_2}$, C-14, C-17), 47.25-48.04 (C-15 and C-16), 66.91 ($\underline{CH_2}$Phe), 69.63 ($O\underline{CH_2}CH_2$), 69.45 (C-2), 70.57 (C-5), 70.85 (C-4), 71.44 (C-3), 96.25 (C-1), 128.43 (Phe), 156.21 and 171.30 (CO).

g) Synthesis of 15-octadecylaminopentadecanyl 2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranoside

Palladium on active charcoal at 10% (0.5 g) under hydrogen pressure is added to a solution of 15-[N-(benzyloxycarbonyl)octadecylamino]pentadecanyl 2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranoside obtained in the preceding step f) (2.72 g; 4.23 mmol) in methanol (100 ml). The reaction is quantitative.

^{1}H NMR: δ (ppm) 0.87 (t, 3H, *J= 6.96* Hz, H-33), 1.2 (d,

3H, *J= 6.51* Hz, H-6), 1.25 (m, 54H, $(CH_2)_{27}$), 1.52 (m, 6H, $OCH_2\underline{CH_2}$, H-14 and H-17), 1.88-1.93 (band NH), 1.95, 2.05 and 2.15 (s, 3H, $OCO\underline{CH_3}$), 2.64 (m, 4H, H-15 and H-16), 3.46 (m, 1H, $O\underline{CH_a}CH_2$), 3.63 (m, 1H, $O\underline{CH_b}CH_2$), 3.79 (m, 1H, H-5), 4.41 (d, 1H, *J= 7.98* Hz, H-1), 4.99 (dd, 1H, *J= 3.52* Hz and *10.46* Hz, H-3), 5.16 (dd, 1H, *J= 7.98* Hz and *10.46* Hz, H-2), 5.23 (dd, *J= 3.52* Hz and *3.31* Hz, H-4).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.68 (C-33), 17.31 (C-2), 20.75 ($\underline{CH_3}COO$), 27.29 (C-6), 29.72 ($(CH_2)_{27}$)), 25.89-31.98 ($OCH_2\underline{CH_2}$, C-14, C-17), 47.75-48.04 (C-15 and C-16), 69.63 ($O\underline{CH_2}CH_2$), 69.45 (C-2), 70.57 (C-5), 70.85 (C-4), 71.44 (C-3), 96.25 (C-1), 171.30 (CO).

h) Synthesis of (3-[4-(3-aminopropylamino)butylamino-propylbenzyloxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecanyl 2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranoside

Diisopropylethylamine (0.491 ml; 2.82 mmol), BOP (0.457 g; 1.03 mmol) and {3-[4-(3-benzyloxycarbonylaminopropylbenzyloxycarbonyl-amino)butylbenzyloxycarbonylamino]propylamino}acetic acid obtained in step a) (0.748 g; 0.94 mmol) are added successively to a solution of 0.60 g of the compound obtained in the preceding step g) (0.94 mmol) in chloroform (15 ml). The resulting oil is purified by chromatography (4:6 heptane/ethyl acetate).

(3-[4-(3-aminopropylamino)butylaminopropylbenzyl-oxycarbonylamino]methylenecarbamoyl)-15-pentadecanyl-

16-octadecanyl 2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranoside is obtained with a yield of 45%.

^{1}H NMR: δ (ppm) 0.87 (t, 3H, *J= 6.96* Hz, H-33), 1.2 (d, 3H, *J= 6.51* Hz, H-6), 1.24 (m, 54H, $(CH_2)_{27}$), 1.39-1.67 (m, 15H, $OCH_2\underline{CH_2}$, H-14, H-17, NH, CH_2), 1.95, 2.05 and 2.15 (s, 3H, $OCOCH_3$), 3.05-3.35 (m, 18H, H-15, H-16 and $\underline{CH_2}N$), 3.43 (m, 1H, $O\underline{CH_a}CH_2$), 3.67 (m, 1H, *J= 6.74* Hz, $O\underline{CH_b}CH_2$), 3.79 (m, 1H, H-5), 4.41 (d, 1H, *J= 7.98* Hz, H-1), 4.99 (dd, 1H, *J= 3.52* Hz and *10.46* Hz, H-3), 5.05 (s, 8H, $\underline{CH_2}$Phe), 5.16 (dd, 1H, *J= 7.98* Hz and *10.46* Hz, H-2), 5.23 (dd, *J= 3.52* Hz and *3.31* Hz, H-4), 5.47 (band CONH, 1H), 7.32 (m, 20H, Phe).

^{13}C NMR ($CDCl_3$): δ (ppm) 14.84 (C-33), 20.75 ($\underline{CH_3}COO$), 27.29 (C-6'), 29.72 ($(CH_2)_{27}$), 25.89-31.98 ($OCH_2\underline{CH_2}$, C-14, C-17 and CH_2), 37.87-46.87 (C-15, C-16 and C-N), 66.84 ($\underline{CH_2}$Phe), 68.63 ($O\underline{CH_2}CH_2$), 69.45 (C-2), 70.57 (C-5), 70.85 (C-4), 71.44 (C-3), 96.25 (C-1), 128.31 (Phe), 157.01 and 171.30 (CO).

i) Synthesis of 1-[-(3-[4-(3-aminopropylamino)-butylaminopropylbenzyloxycarbonylamino]-methylenecarbamoyl)-15-pentadecanyl-16-octadecanyl 6-deoxy-β-L-galactopyranoside

A saturated methanolic solution of ammonia (1 ml) is added to a methanolic solution (3 ml) containing the product obtained in the preceding step h) (0.60 g; 0.94 mmol). After one hour, the mixture is concentrated.

^{1}H NMR: δ (ppm) 0.87 (t, 3H, *J= 6.96* Hz, H-33), 1.2 (d,

2H, *J= 6.51* Hz, H-6), 1.24 (m, 54H, $(CH_2)_{27}$), 1.39-1.67 (m, 15H, $OCH_2\underline{CH_2}$, H-14, H-17, NH, CH_2), 3.05-3.35 (m, 18H, H-15, H-16 and $\underline{CH_2}N$), 3.4-3.7 (m, 6H, $O\underline{CH_2}CH_2$, H-3, H-4, H-5, H-2), 4.73 (d, 1H, *J= 7.98* Hz, H-1), 5.05 (s, 8H, $\underline{CH_2}$Phe), 5.47 (band CONH, 1H), 7.32 (m, 20H, Phe).

j) Synthesis of 1-[-(3-[4-(3-aminopropylamino)butyl-aminopropylamino]methylenecarbamoyl)-15-pentadecanyl-16-octadecanyl 6-deoxy-β-L-galactopyranoside (compound 3)

Palladium on charcoal at 10% (0.032 g) in methanol is added to a solution of the product obtained in the preceding step i) (0.072 g; 0.05 mmol). After one night, the mixture is filtered through fiber glass paper and concentrated in a rotary evaporator. The product is then purified by HPLC on a C-4-type preparative column.

^{1}H NMR: δ (ppm) 0.87 (t, 3H, *J= 6.96* Hz, H-33), 1.2 (d, 2H, *J= 6.51* Hz, H-6), 1.24 (m, 54H, $(CH_2)_{27}$), 1.39-1.67 (m, 15H, $OCH_2\underline{CH_2}$, H-14, H-17, CH_2), 2.92-3.19 (m, 18H, H-15, H-16 and $\underline{CH_2}N$), 3.4-3.7 (m, 6H, $O\underline{CH_2}CH_2$, H-3, H-4, H-5, H-2), 4.73 (d, 1H, *J= 7.98* Hz, H-1).

C\USE OF THE TRANSFER AGENTS ACCORDING TO THE INVENTION

Example 4: Preparation of transfer agent/nucleic acid complexes with compound 2 and measurement of their size

This example illustrates the preparation of complexes between a transfer agent according to the

invention and a nucleic acid, their size having then been measured.

The glycolipid used in this example and in the examples which follow is compound 2, dissolved in chloroform, at a concentration of 10 mg/ml. In some cases, a neutral co-lipid, cholesterol or DOPE, was mixed with compound 2 beforehand.

The lipid solution is prepared in the following way: a sample of the desired amount is collected, the solvent is evaporated over an argon stream and left to dry for 1 hour. The lipid is then rehydrated with a solution containing 5% dextrose and 10 mM sodium chloride overnight at 4°C. The following day, the lipid solutions are heated at 60°C for 5 minutes and then treated with ultrasound for 1 minute. The operation is repeated until the size of the lipid particles is stable.

The DNA used is the plasmid pXL3031 (figure 1) dissolved in a mixture of 5% dextrose and 10 mM sodium chloride at a concentration of 0.5 mg/ml or of 1.0 mg/ml. This plasmid contains the luc gene encoding luciferase under the control of the P/E CMV promoter of cytomegalovirus. It is 3671 bp long. The diagram of this plasmid is represented in figure 1. The plasmid pXL3031 was purified according to the methods described in patent application WO 97/35002.

The compound 2/DNA complexes are prepared by rapidly mixing suitable volumes of solution of plasmid

DNA and of compound 2 (according to the desired charge ratio), at room temperature. The amount of transfecting agent varies between 0.25 nmol/µg of DNA and 12 nmol/µg of DNA.

The size of the complexes was analyzed by measuring the hydrodynamic diameter by dynamic laser light scattering using a Coulter N4Plus machine. The samples are diluted 20-fold in a solution containing 5% dextrose and 20 mM of sodium chloride in order to avoid multiple scattering.

At a ratio of 3 nmol of lipid/µg of DNA, the following results were obtained:

	Size in nm
Micelles	130 nm
Formulation with cholesterol	153 nm
Formulation with DOPE	137 nm

The term "micelles" indicates that compound 2 was used alone, i.e. without adding neutral co-lipid, and it therefore forms a micellar solution.

This table shows that the complexes obtained have a size between 130 nm and 150 nm approximately, which is compatible with pharmaceutical use, in particular by injection.

Example 5: Behavior of the complexes formed from compound 2 at various charge ratios

This example illustrates the behavior of the transfer agent according to the invention/nucleic acid complexes when the charge ratio is varied. The impact of adding a co-lipid (cholesterol or DOPE) is also illustrated.

Conventionally, 3 physicochemical phases are distinguished when the transfer agent/DNA charge ratio is increased (B. Pitard et al., *Virus-sized self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer*, PNAS, Vol. 94, pp. 14412-14417, 1997). These three phases determine the therapeutic potential of the transfer agent.

At a low charge ratio, the DNA is not saturated with the transfer agent. Noncomplexed DNA still remains, and the complexes are negatively charged overall and small in size. This phase, which is stable, is called "Phase A".

The fact that the DNA is not completely saturated with the transfer agent means that the DNA is not completely protected. The DNA can therefore be subjected to degradation by nucleases. Moreover, since the complexes are negative overall, the crossing of the cell membrane is difficult. For these reasons, the nucleolipid complexes of phase A are relatively inactive.

At an intermediate charge ratio, the DNA is completely saturated with the transfer agent, and the complexes are neutral or slightly positive overall.

This phase is unstable since the ionic repulsions are minimal and an aggregation phenomenon can occur. The size of the particles is well above the limit of detection by dynamic laser light scattering (much greater than 3 μm). This unstable phase is called "phase B". Such a complex size is not suitable for uses by injection, although that does not mean that the complexes are inactive in phase B: they are merely in a formulation which is not suitable for injecting them for a pharmaceutical purpose.

At a higher charge ratio, the DNA is supersaturated with the transfer agent, and the complexes are positive overall. Because of the strong repulsions between the positive charges, this phase is stable. It is referred to under the name "phase C". Unlike phase A, the complexes obtained are in a form such that the DNA is very well protected against nucleases, and the overall positive charge of these complexes facilitates attachment to the cell membrane which is anionic in nature and the crossing of this membrane. The complexes of phase C are therefore particularly suitable for use for transferring nucleic acids into cells.

These 3 zones A, B and C were also updated with compound 2 according to the invention as the transfer agent:

Charge ratio	0.25	0.5	0.75	1	1.5	2	3	4	6	8	10	12
Micelles	A	A	**B**	**B**	**B**	**B**	C	C	C	C	C	C
+Chol-esterol	A	A	**B**	**B**	**B**	C	C	C	C	C	C	C
+DOPE	A	A	**B**	**B**	**B**	C	C	C	C	C	C	C

As shown in the table above, zone B, which is the instability zone, is particularly small and occurs at very low charge ratios. Zone C begins from 2 nmol of lipid/μg of DNA when compound 2 is used together with a co-lipid (cholesterol or DOPE), and from 3 nmol of lipid/μg of DNA when the compound is used alone. As specified above, this is the zone in which it is particularly advantageous to operate for pharmaceutical use.

By way of comparison, it has been shown, with one of the cationic lipids disclosed in patent WO 97/18185, that zone C begins to form at charge ratios at least equal to 2, depending on the sodium chloride concentration of the solution (see figure 3A in B. Pitard et al., PNAS USA, 94, pp. 14412-14417, 1997).

Thus, compound 2 is a particularly advantageous transfer agent since it is stable at low charge ratios, which makes it possible to form stable complexes with small amounts of glycolipids, with the

beneficial consequences which ensue therefrom from a toxicity point of view.

Example 6: Use of compound 2 for *in vitro* DNA transfer

This example illustrates the capacity of the transfer agents according to the invention to transfect DNA into cells *in vitro*, at various charge ratios, in the presence and absence of a neutral co-lipid (cholesterol or DOPE).

24-well microplates are seeded with 60,000 HeLa cells per well, and are grown overnight. The number of cells after one night, and therefore at the time of transfection, is 100,000 cells per well.

Each well is brought into contact with the complexes formed with compound 2 and containing 1 µg of plasmid DNA in 0.5 ml of DMEM culture medium (Gibco/BRL without serum. The cells are incubated at 37°C for 5 hours. The medium containing the complexes is then removed and replaced with a DMEM culture medium containing 10% of fetal calf serum. Then, the cells are again cultured for 24 hours. Finally, the cells are lysed and assayed using a luciferase assay kit (Promega) and a Dynex MLX luminometer.

The results obtained are indicated on the histogram of figure 2. The transfer efficiency is represented by the luciferase expression in pg/well. It is noted that the maximum transfection is approximately 500 pg/well.

In conclusion, this example clearly shows that it is possible to use compound 2 according to the invention to form complexes capable of promoting DNA transfer into cells *in vitro*.

Example 7: Use of compound 2 for *in vivo* DNA transfer

This example illustrates the capacity of the transfer agents according to the invention to transfect DNA into cells *in vivo*.

The *in vivo* gene transfer was carried out on Balb/C mice by intratracheal, intravenous and intramuscular administration.

In the case of intramuscular injections, each mouse received 30 µl of formulation containing 15 µg of plasmid DNA, in the anterior muscle of the tibia. The tissues are recovered 7 days after injection, and they are frozen and stored at -80°C while waiting to carry out the luciferase activity assays.

In the case of intravenous injections, each mouse received 200 µl of formulation containing 50 µg of plasmid DNA. The tissues are recovered 24 hours after injection, and are then frozen and stored in the same way as above.

Figure 3 illustrates the activity of the complexes formed with compound 2, for *in vivo* gene transfer by intramuscular injection. These results clearly show that the formation of complexes with compound 2 according to the invention and DNA makes it

possible to promote the transfer of said DNA into cells *in vivo*.

Similarly, it is possible to use any transfer agent as defined in the present invention, to promote DNA transfer into the cells of any type of tissue.